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Role of epicardium-derived Pw1 cells in heart homeostasis and growth during pregnancy

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Abstract

Imprinted genes in placental mammals play a critical role in regulating prenatal growth and setting the metabolic rate of development. Highly expressed in embryos and early postnatal life, imprinted genes are down regulated when the organism reaches adulthood, remaining expressed in individual cells where they control activation and plasticity. Maternally imprinted gene Pw1, also known as Peg3, recently was described as a potential adult stem cell marker in various tissues such as bone marrow, central nervous system and intestines. In skeletal muscle it marks myogenic stem cells, which are located in interstitia and can efficiently contribute to muscle regeneration after focal freeze-crush injury. Using a transgenic Pw1:nLacZ reporter mouse, I described a subpopulation of proepicardial/epicardial derived mesenchymal cells involved in maintaining homeostasis of adult mouse heart through secretion of various growth factors. Immunohistochemistry and cytometric analysis revealed that myocardial Pw1 cells express the cardiac stem cell membrane receptors Sca1, Pdgfra, Cd34 and Cd29, making them a potentially interesting component of regenerative medicine for further investigation. Based on mRNA expression profiles and patterns during embryonic development I surmise an epicardial origin of Pw1 cells and their involvement in cardiac growth, as adult Pw1 cells are highly pro-angiogenic in vitro and can induce tube formation when they are co-cultured with an endothelial cell line. To investigate the role of Pw1 cells during postnatal cardiac growth I have linked their action to heart hypertrophy during pregnancy. Using pharmacological tools I locally depleted Pw1 cells which resulted in local cardiomyocyte atrophy and fibrosis. To explore the remodelling potential of Pw1 cells in pathological conditions we are planning to investigate their involvement in disease models of myocardial infarction.
Zusammenfassung

Geprägte Gene in Plazentatiere spielen eine entscheidende Rolle bei der Regulierung des pränatalen Wachstums und der Einstellung der Stoffwechselrate während der Entwicklung. Gene, die im embrionalen und frühen postnatalen Stadium hoch exprimiert sind, werden im Erwachsenenstadium herunterreguliert und bleiben nur noch in einzelnen Zellen exprimiert, in denen sie die Aktivierung und Plastizität kontrollieren.

Das mütterlich geprägte Gen Pw1, auch als Peg3 bekannt, wurde vor kurzem als potentieller adulter Stammzellmarker in verschiedenen Geweben wie dem Knochenmark, dem zentralen Nervensystem und dem Darm, beschrieben. Im Skelettmuskel markiert er myogene Stammzellen, die sich im Interstitium befinden und die effizient zur Regenerierung der Muskulatur nach einer fokalen Gefrier-Quetschverletzung beitragen können.

Mit der transgenen Pw1:nLacZ Reportermaus, haben wir eine Subpopulation von proepikardial / epikardialen mesenchymalen Zellen beschrieben, welche an der Aufrechterhaltung der Homöostase des adulten Mausherzens durch Sekretion verschiedener Wachstumsfaktoren beteiligt sind. Die Immunhistochemie und zytometrische Analyse ergab, dass die miokardialen Pw1 Herzstammzellen die Zellmembran-Rezeptoren Sca1, Pdgfra, Cd34 und Cd29 exprimieren, wodurch sie ein interessantes Ziel weiterer Untersuchungen im Rahmen der regenerativen Medizin sind. Basierend auf mRNA-Expressionsprofilen und Mustern während der Embryonalentwicklung vermuten wir einen epikardialen Ursprung der Pw1 Zellen und ihre Beteiligung am Herzwachstum. Adulte Pw1 Zellen sind stark proangiogen in vitro und können die Enstehung von Gefäßen induzieren, wenn sie zusammen mit einer endothelialen Zelllinie ko-kultiviert werden. Um die Rolle von Pw1 Zellen während des postnatalen Herzwachstums zu untersuchen, wurde ihre Funktion mit der Hypertrophie des Herzens während der Schwangerschaft in Verbindung gesetzt. Mit pharmakologischen Instrumenten haben wir lokal Pw1 Zellen entnommen, welches zu einer lokalen Atrophie und Fibrose der Kardiomyozyten führte. Um das Umbaupotenzial der Pw1 Zellen in pathologischen Bedingungen zu
untersuchen, planen wir ihre Beteiligung in verschiedenen Krankheitsmodellen des Myokardinfarkts zu erforschen.
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<tbody>
<tr>
<td>7AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>hEND</td>
<td>Brain endothelial</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BW</td>
<td>Body weight</td>
</tr>
<tr>
<td>CAB</td>
<td>Chicken beta actin</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre-recombinase</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Eagle’s minimal essential medium</td>
</tr>
<tr>
<td>DMR</td>
<td>Differentially methylated region</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>EPDC</td>
<td>Epicardium-derived cell</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FACs</td>
<td>Fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluorescein di(β-D-galactopyranoside)</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin stain</td>
</tr>
<tr>
<td>ICR</td>
<td>Imprinting control region</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IGN</td>
<td>Imprinted gene network</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LT-HSC</td>
<td>Long term HSC</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>nLacZ</td>
<td>Mouse embryonic fibroblast cell line</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PICS</td>
<td>PW1+/Pax7- interstitial cells</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>TA</td>
<td>Tibialis anterior muscle</td>
</tr>
<tr>
<td>Tb4</td>
<td>Thymosin β4</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition/Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney Cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HW</td>
<td>Heart weight</td>
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</table>
1. Introduction

In the fourth century BC, Greek philosopher Aristotle in his *Historia Animālium* described the beating heart as a first organ to be formed in chicken embryos. In formation of the beating heart he saw a starting point for the body plan and as such a beginning of life itself. During classical antiquity the heart was not only considered to be a “seed of life”, but also a chamber that hosts the soul. From the Aristotle’s time, through the middle ages, up to the modern days heart remains one of the most studied organs. In the past few decades we have learned a great deal about heart’s embryonic development and about the mechanical force of the cardiomyocyte, but it still remains unclear how different cell types regulate and maintain the beating heart.

The heart is a muscle that supplies the rest of the body with nutrients and oxygen and because of this important role the heart is also an organ whose physiological state is reflected in the total physiological state of the organism and vice versa. According to the 2012 WHO report (http://www.who.int/gho/publications/world_health_statistics/2012/en/index.html) the leading cause of death in developed world is congestive heart failure, most frequently caused by myocardial infarction (MI) (48%). The damaged area of the heart in patients who survive MI is scarred permanently, causing future MIs, eventually leading to complete organ failure. Furthermore it is projected that the number of deaths due to cardiovascular disease will almost double until 2030. For these reasons heart repair and heart regeneration are a main focus of regenerative medicine. Today’s regenerative medicine efforts for heart repair could be divided in three different strategies: promoting heart repair via activation of cardiac resident stem cells (Smart et al., 2011; Beltrami et al., 2003; Oh et al., 2003); transplantation of *in vitro* derived cardiomyocytes (Qian et al., 2012; Radisic et al., 2007); or administrating various growth factors and compounds such as IGF1 (Santini et al., 2007; Padin-Iruegas et al., 2009) or NRG1 (Bersell et al., 2009) that promote cardiomyocyte proliferation, improving vascularisation and modulating immune response to prevent scar formation. Even though the progress of understanding processes involved in heart repair has been made, we still
haven’t developed successful therapy for life improvement for millions of patients.

In recent years powerful insights into functional heart regeneration came from studies conducted in lower vertebrates, such as zebrafish or newts which re-grow functional myocardial tissue after partial amputation of the heart and repair damaged areas without the generation of non-functional scar tissue (Poss et al, 2002; Bettencourt-Dias et al., 2003; Laube et al., 2006; Singh et al., 2010). New muscle comes from partial dedifferentiation and proliferation of already existing cardiomyocytes and mobilization of epicardial cells. Recently it was shown that mechanisms and pathways involved in successful heart regeneration in lower vertebrates are also present in mammalian heart (Figure 1). Porrello and colleagues (2011, 2013) described the remarkable regenerative capacity of the neonatal murine heart. Newborn mice (up to seven days) subjected to 10-25% of ventricular amputation could regenerate in a very similar way to zebrafish through dedifferentiation and proliferation of pre-existing cardiomyocytes. Mice restored complete heart function after 30 days without showing any scaring and signs of pathological hypertrophy. Those results are revealing that the regenerative process is more conserved than we previously thought.

Other studies also changed our view of adult heart as a terminally differentiated organ. There is compelling evidence that the adult human heart possesses populations of cardiomyocytes with proliferative potential (Beltrami et al., 2001) and furthermore human cardiomyocytes renew around 1% until age 25 (Bergmann et al.; 2009). Recently similar results were also found in ageing mice by measuring the incorporation ratio between $^{15}$N and $^{14}$N labelled thymidine (Senyo et al., 2013). These results suggest that the lack of cardiomyocytes capable of proliferation is not the main reason for the poor regenerative capacity of mammalian heart.

From zebrafish we know that other cardiac cell types, such as epicardium, have a crucial role in heart regeneration, development and homeostasis through promoting vascularisation, but it remains unclear what is the role of these cells in remodelling of the mammalian heart in physiological and pathological conditions.
Figure 1. Cardiac regeneration in zebrafish and neonatal mice

(A) Partial resection of ventricular myocardium of zebrafish leads to complete regeneration of cardiac muscle within 4-8 weeks without any signs of fibrosis (adapted from Poss et al., 2002). (B) Similar regenerative response characterized by cardiomyocyte proliferation with minimal hypertrophy or fibrosis is described in 1-day-old neonatal mice (adapted from Porrello et al., 2011). This remarkable regenerative capacity is lost by 7 days of age.

Role of epicardium in heart development, homeostasis and regeneration

The epicardium is the outer surface of the heart and consists of a single epithelial layer and underlying connective tissue. Blood vessels and nerves that supply the heart lie in the epicardium and are surrounded by adipose tissue that cushions the heart in the pericardial cavity.

During development, the epicardium derives from the proepicardium and it has been shown that a population of epicardial cells goes through epithelial to mesenchymal transition (EMT), migrates into the developing
myocardium and gives rise to the vasculature and interstitial cells (Figure 2). This process is mandatory for normal heart development and impairment by deletions of selected genes expressed in the epicardium (like Raldh2 and Tcf21) or signalling necessary for EMT and invasion (like thymosin beta 4 and Fgfr1) leads to severe defects (Kikuchi et al., 2011; Mahtab et al. 2008, Smart et al., 2007; Pennisi and Mikawa 2009). Epicardial derived cells (EPDCs) after invading the myocardium can differentiate into several heart populations, including fibroblasts, pericytes, smooth muscle and endothelial cells (Winter et al., 2007; Wilm et al., 2005; Azambuja et al., 2010). Zhou and colleagues (2008) also showed that mouse EPDCs could have cardiomyocyte potential. Using Cre recombinase driven by the Wt1 promotor (transcription factor located within epicardium) they showed that during normal murine heart development a subset of epicardial cells differentiate into fully functional cardiomyocytes. Although epicardium as a source of cardiac progenitors was also described in adult heart after MI (Smart et al., 2011; Huang et al., 2012), it is still very controversial and needs further investigation. A major problem impeding genetic fate mapping is the lack of specific epicardial markers. Lineage tracing based on regulatory sequences from Tbx18 and Wt1 is not reliable because their expression is not restricted only to epicardial progenitors. Using Tcf21 (epicardin) as a more specific marker of epicardium in zebrafish, it was found that epicardial cells do not contribute to myocardium (Kikuchi et al., 2011). In general the rate of cardiomyocyte generation from epicardium is very modest.

In the adulthood, as in development, mammalian epicardium is much more than an outside cover of the heart. Epicardial progenitors with multilineage potential persist in adulthood in mice and humans (Smart et al., 2007; van Tuyn et al., 2007). Chong and colleagues (2011) described a population of EPDCs based on expression of PDGFRa that occupy perivascular and adventitial niches. Using in vitro system they showed that these subpopulations could be differentiated into smooth muscle and endothelial cells, cardiomyocytes, hepatocytes and neurons. This trans-germ layer potency makes epicardial cells and EPDCs interesting tool for regenerative medicine.
Figure 2. Model for the role of epicardium in coronary vessel development

During normal heart development epicardial cells undergo an epithelial to mesenchymal transition (EMT) in response to BMP, FGF, TGFβ and Tβ4 and migrate into developing myocardium where they respond to angiogenic factors (like VEGF and PDGF) and differentiate into smooth muscle or endothelial cells (adapted from Smart et al., 2007).

In zebrafish it has been shown that regeneration of the injured heart proceeds through two coordinated steps in which epicardium plays an important role (Lepilina et al., 2006). First a blastema of progenitor cells is formed at the apical edge of the existing myocardium. Then epicardial tissue surrounding both cardiac chambers induces developmental markers and rapidly expands, creating a new epithelial cover for the exposed myocardium. Some of these epicardial cells undergo EMT, in similar way to that in development, and invade the wound and provide new vasculature and signals to regenerating myocardium. Although epicardial cells do not contribute to the
new cardiac muscle after injury, blocking the activation or migration of epicardium leads to fibrosis rather than regeneration.

Smart and colleagues (2007) suggest that one of the reasons why mammalian heart cannot regenerate well is because the epicardium cells don’t have migrating potential and cannot form vasculature in newly formed myocardial tissue. They also showed that thymosin β4 (Tβ4) could promote vessel formation in mice by recruiting epicardial cells, which would have considerable therapeutic potential in humans. Priming the adult mouse heart with Tβ4 reactivates epicardial Wt1 and induces their migration into myocardium (Smart et al 2011). Furthermore after experimental MI not only did animals treated with Tβ4 showed better recovery, but also Wt1 cells contributed to myocardial mass.

Recent studies suggest EPDCs are not a major source of cardiac progenitors but their important role comes from secretion of trophic factors such as retinoic acid (RA) (Chen et al., 2002), which stimulate growth and morphogenesis of developing myocardium. Formation of epicardium and migration of foetal EPDCs corresponds to thin-walled ventricular chamber expansion and formation of compact myocardium. One of the signals involved in compact zone formation and cardiomyocyte proliferation is retinoic acid. Rat embryos deficient in RA (Wilson and Warkany, 1949), mouse embryos lacking the retinoic acid receptor gene RXRa (Sucov et al., 1994; Kastner et al., 1994) and mouse embryos lacking the RA synthetic enzyme Raldh2 (Brade et al., 2011) all suffer from an inability to properly form the compact zone (Figure 3). Chen and co-workers (2002) showed that foetal epicardium responds to retinoic acid by secreting trophic factors, which stimulate cardiomyocyte proliferation and compact zone morphogenesis.

One of the most prominent morphogens secreted in response to retinoic acid (RA) signalling by embryonic mouse epicardium is IGF2 (Brade et al., 2011; Li et al., 2011). Brade and colleagues (2011) proposed a new mechanism for RA-mediated myocardial expansion in which RA directly induces hepatic EPO resulting in activation of epicardial IGF2 that stimulates compact zone growth. Epicardial IGF2 is required for ERK proliferation pathway in the developing heart and disruption of IGF signalling resulted in a
significant decrease in cardiomyocyte proliferation and ventricular wall hypoplasia (Li et al., 2011).

Figure 3. Comparison of heart phenotype in rescued Raldh2/- and Rxra/- mouse embryos
(A-F) Sections of H&E stained E13.5 hearts from wild-type (A,D), germline Rxra/- (B,E) and rescued Raldh2/- (C,F) embryos. Representative heart sections are shown at 40× magnification in A-C and detailed photographs of the compact zone at 400× magnification in D-E. Both mutants exhibit a severe reduction in size of the compact zone myocardium (CZ) whereas the trabecular myocardium (Tr) is only mildly affected; the black bar indicates the thickness of the compact zone. Similar results were observed for all mutants analyzed (n=3 for each genotype). Rescued Raldh/- embryos refer to low dose treatment with RA which delays lethality from E9.5 to E13.5. Taken from Brade et al (2011).

Many other specific growth factors secreted from the epicardium are responsible for heart morphogenesis during development (Figure 4). Members of the fibroblast growth factor (FGF) family are involved in ventricular development, as mouse knockouts of ligand or receptor genes result in a hypoplastic phenotype (Lavine et al., 2005). Erythropoietin (EPO)
and EPO receptor (EPOR) are highly expressed in the epicardium and both EPO and EPOR null embryos display a thin ventricular wall with abnormal epicardial structure, which appears often detached from the myocardium (Wu et al., 1999). Furthermore EPO works with RA to promote cardiomyocyte proliferation. Blocking of EPO signalling with ant-EPOR antibodies results in decreased cardiomyocyte proliferation that could be rescued by exogenous RA administration (Stuckmann et al., 2003). During coronary vessel development TGFβ ligands are detected both in the epicardium and myocardium (Molin et al., 2003) and ablation of TGFβ3R results in thinner epicardium, detached from the myocardium and a partial disruption of coronary vessels (Compton et al., 2007).

Several Wnts and their receptors are expressed in the developing heart. Canonical Wnt signalling inhibits cardiac differentiation and maintains secondary heart field in an undifferentiated state. On the other hand, Non-canonical Wnt signalling promotes cardiomyocyte differentiation and is required for outflow tract development (Cohen et al., 2008). Specific deletion of β-catenin in the proepicardial organ leads to myocardial hypoplasia and embryonic lethality between E15.5 and birth. Finally, β-catenin, Wnt9b and FGF2 are retinoid-induced growth factors since they are down-regulated in epicardial explants from epicardial RXRα knockout mice (Merki et al., 2005). During heart development Sonic hedgehog (Shh) is expressed in the epicardium and is responsible for expression of multiple proangiogenic molecules, including VEGFA, VEGFB, VEGFC and angiopoietins in a FGF dependent manner (Lavine et al., 2006).

Besides representing a source of progenitors, adult EPDCs also could play an important role through secretion of growth factors that promote angiogenesis and cardiac survival (Zhou et al., 2011). To test proangiogenic role of EPDCs in post-MI heart, cells were isolated from infarcted heart based on Wt1 expression and expanded in culture. A large number of proangiogenic transcripts were highly enriched in EPDCs, including Vegfa, Angpt1, Ang, Fgf1, Fgf2, Fgf9, Pdgfa, Pdgfc, Pdgfd, Adamts1, Sdf1, Mcp1, and Il6. The EPDC conditioned media stimulated growth of multiple types of endothelial cells in vitro and reduced infarct size and improved heart function in vivo. Treating the conditioned media with blocking antibodies for FGF2 and VEGFA
accounted for approximately half the growth-promoting activity on endothelial cells.

**Figure 4. Growth factor mediated signalling pathways involved in myocardial and coronary vessel growth in the embryonic heart.**

Progenitor cells in the epicardium give origin to epicardial cells and cardiomyocytes. BMP/FGFs signalling plays a role in the cardiogenic commitment of proepicardial cells. Signalling pathways involving RA, Shh, VEGF and Ang2 are required for endothelial cell (ECs) differentiation of epicardial cells. TGFβ, Thymosin β4, Wnt/β-catenin and PDGF signalling are involved in regulating epicardial cell differentiation into smooth muscle cells (SMCs). A crosstalk between epicardial and myocardial growth factor signalling is also shown. RA regulates FGF and Shh expression from the epicardium modulating both epicardial and myocardial cell functions and induces VEGF and Ang2 expression from myocardial cells. Taken from Limana et al (2011).
A recent study from Huang and co-workers (2012) showed that the injury of the adult heart results in activation of developmental genes in the epicardium through C/EBP transcription factors. Disruption of C/EBP signalling in the adult epicardium resulted in reduced fibrosis and enhanced cardiac function through reduction of inflammation in the first few days after injury.

These recent results describe EPDCs as cells that in one hand promote heart healing through secretion of survival factors like VEGF and FGF2 (Zhou et al., 2011), and in the other are responsible for prolonging the pro-inflammatory phase (Huang et al., 2012) in post-injured heart. These contradictory results show the importance of epicardium during heart healing by promoting cardiomyocyte survival, angiogenesis and by regulating inflammatory response and neutrophil infiltration through still unknown temperospatial mechanisms and pathways.

One of the problems of studying epicardium is the lack of appropriate epicardium specific markers. Most common markers such as Wt1, Tbx18 or Tcf21 are not restricted only to epicardium and EPDCs, and are not expressed in the entire structures (epicardium/proepicardium) or lineages. Wt1 encodes a zinc-finger transcription factor important for epicardial cell adhesion and for maintaining high levels of Raldh2 expression (Moore et al., 1999; von Gise et al., 2011; Guadix et al., 2011). It is found in epicardium, in the subepicardial mesenchyme and in migratory EPDCs during development (Carmona et al., 2001) During epicardial differentiation Wt1 expression is down regulated and in post-natal heart is barely detectable (Perez-Pomares et al., 2002). Using two cre knock-in alleles of Wt1 (Wt1CreEGFP and Wt1CreERT2) several groups postulated epicardial contribution to the myocardium, interventricular septum, fibroblasts, smooth muscle and endothelial cells (Zhou et al., 2008; Smart et al., 2011) Furthermore, using the same two Wt1: Cre lines Smart and colleagues (2011) demonstrated epicardial contribution to newly formed myocardium after myocardial infarction with thymosin β4 pretreatment. Rudat and Kispert (2012) disputed these findings by using in situ hybridization and immunofluorescence on tissue sections. They detected endogenous expression of Wt1 mRNA and protein in the endothelial cells throughout cardiogenesis and moreover using the same Wt1:Cre lines.
mentioned before they described Cre activity in cardiomyocytes before establishment of proepicardium/epicardium.

Although Tbx18 expression is largely restricted to proepicardial organ during early heart development, at later stages in a similar manner as Wt1 endogenous Tbx18 is also found in a subpopulation of cardiomyocytes and mesenchymal progenitors (Haenig and Kispert, 2004; Torlopp et al., 2010; Christoffels et al., 2009). A more restricted epicardial marker during heart development is Tcf21, also known as epicardin (Quaggin et al., 1998; Braitsch et al., 2012). Cell fate analysis in the zebrafish using Tcf21<sup>Cre</sup> showed that epicardial cells are limited to fibroblasts and smooth muscle cells (Kikuchi et al., 2011). In the postnatal heart Tcf21 mostly is expressed in interstitial fibroblasts and is not expressed in epicardium, smooth muscle or endothelial cells (Acharya et al., 2011). The proepicardial organ and epicardium consist of several cell populations whereas Wt1, Tbx18 and Tcf21 are expressed only in subpopulation of proepicardial cells thus making them inadequate tools for epicardial studies (Braitsch et al., 2012; Katz et al., 2012, Russell et al., 2011).

These findings suggests that frequently used Cre-mediated lineage tracing systems (Wt1<sup>CreEGP</sup>, Wt1<sup>CreERT2</sup>, Tbx18<sup>Cre</sup> and Tcf21<sup>Cre</sup>) are not suitable for epicardial fate mapping because of endogenous expression in other cell lineages, ectopic or poor recombination efficiency and expression only in sub-lineage of epicardial cells. In this study we show that a recently discovered adult pan-stem cell marker Pw1 identifies subpopulations of epicardial cells and EPDCs in the adult mouse heart. The main focus of this research was to identify Pw1 cells in adult mouse heart and to investigate their role in cardiac homeostasis. The potential use of Pw1 as a marker of epicardium and EPDCs during development and adulthood warrants further investigation.
Paternally expressed gene Pw1/Peg3 regulates metabolic rate of embryonic development and postnatal growth in mammals

The imprinted genes were discovered in early 1980s as a result of two types of mouse experiments: generating uniparental embryos using nuclear transplantation (McGrath and Solter, 1984; Barton et al., 1984) and using genetic techniques to make embryos that inherited specific chromosomes from one parent only (uniparental disomy) (Cattanach and Kirk, 1985). In both cases mammalian genes function differently depending on whether they are passed on from father or mother. The imprinted genes play a crucial role in development of placental mammals through regulation of nutrient transfer from the mother to the offspring. Paternally expressed genes generally enhance foetal growth whereas maternally expressed genes suppress foetal growth and the balance between the two is responsible for the setting the normal metabolic rate of development (Moore and Haig, 1991).

Pw1, also known as Peg3, is maternally imprinted Cys2His2 Kruppel-type zinc finger protein (Iuchi et al., 2001; Relaix et al, 1996; Kuroiwa et al., 1996) involved in diverse cellular pathways like cell proliferation (Kohda et al., 2001), p53 mediated cell death (Relaix et al, 2000; Yamaguchi et al., 2002; Deng et al., 2000), inhibition of Wnt signalling by promoting β-catenin degradation (Jiang et al., 2010) and regulation of TNF signalling by activating NFκB (Relaix et al, 1998; Coletti et al., 2002). Furthermore, studies on a mouse knock out model implicated Pw1 in regulating maternal, feeding and male mating behaviour (Li et al, 1999; Curley et al, 2004; Champagne et al, 2009; Swaney et al., 2008). Although from these studies suggest a significant role for Pw1 in various physiological processes, the molecular mechanism through which it acts still remains unclear.

To study the function of Pw1 in vivo Li and co-workers (1999) mutated the Pw1 gene by inserting β-galactosidase (βgeo) selection cassette into its 5’ coding exon using gene targeting. The heterozygous embryos that inherited the Pw1βgeo mutation from paternal germ line showed no detectable wild-type Pw1 mRNA. The paternally transmitted Pw1βgeo heterozygous embryos and
placentas were significantly growth retarded without any histological abnormalities. At birth the mutants were 81\% of the normal weight with proportionally smaller, but morphologically normal organs. Although mice were smaller they were healthy, fertile and normal in their general behaviour. Crossing two paternally inherited Pw1\(^{\beta geo}\) heterozygous mutants revealed a distinct behaviour phenotype. Only 8\% of first litters from mutant mothers survived to weaning age, compared with those nursed by wild type females (83\%). Furthermore progeny of mutant mothers crossed with wild type father also failed to survive, suggesting that father’s genotype was not relevant for this survival rate. Low survival rate was attributed to a maternal nurturing defect. Nurturing defects are connected to reduced hypothalamic oxytocin neurones and increased anxiety-like behaviour that inhibited mothers from exploring pups and resulting in overall poor maternal response (Curley et al., 2004; Li et al., 1999; Champagne et al, 2009).

On the other hand male Pw1\(^{\beta geo}\) mutants have deficits in sexual behaviour and olfactory recognition of sexually receptive mates (Swaney et al., 2007; Swaney et al., 2008). This behaviour phenotype is connected with lower neuronal activity in Pw1\(^{\beta geo}\) mutants. Analysis of forebrain c-Fos expression showed significant sexual experience-dependent changes in neuronal responses to female chemosignals in wild type males but far smaller changes in activation in Pw1\(^{\beta geo}\) males, matching the behavioural findings. According to Swaney and colleagues (2007) postulate that these different behaviours regulated by Pw1 suggests that co-adaptation between individuals has been a key driver in the evolution of imprinting and its major role in mammalian brain evolution.

The imprinting and transcription of the Pw1 gene is regulated by a Pw1-differentially methylated region (DMR) (Kim et al., 2012; Kim et al., 2003). Not only does this imprinting control region (ICR) regulate expression of Pw1, but also the whole cluster of imprinted genes in the Pw1 domain. To investigate the relationship between imprinting and transcription of Pw1 and the genes from the same cluster Kim and co-workers (2012) made a mutant mice in which they knocked-out Pw1-DMR during development. Paternally transmitted mutation caused partial embryonic lethality (50\%) and reduced
body weight. Smaller body weight was much more pronounced in male then female heterozygous pups. Maternally transmitted mutation had no effect on survival and the majority of heterozygous pups were heavier then wild type littermates. Homozygous mutants were never born. On the protein level pups inheriting the knock-out allele paternally showed a 4 fold down regulation of PW1, whereas the maternally inherited allele did not show any changes in PW1 expression.

Figure 5. Schematic summary for the role of the Pw1-DMR on imprinting and expression of Pw1 cluster genes.
The deletion effects of the Peg3-DMR (Pw1-DMR) are schematically presented: each gene is indicated with an arrow while each DMR is indicated with a box. The open and closed boxes indicate unmethylated and methylated state of a given DMR, whereas an X on the box represents the deleted allele (KO) of the Peg3-DMR. The first illustration summarizes the imprinting status of the Peg3 domain that has been known so far (A). The two illustrations below summarize the deletion effects observed from neonatal brains with the paternal (B) and maternal (C) transmission of the KO allele, respectively. Figure was taken from Kim et al (2012).
The Pw1 genomic domain consists of four paternally expressed genes (Zfp264, Usp29, Pw1 and APeg3) and three maternally expressed genes (Zim3, Zim1 and Zim2). As predicted, the deletion of DMR domain caused several changes in expression and imprinting of Pw1- cluster genes depending on the origin of mutation (Figure 5). The paternal transmission of the KO allele in neonatal brain caused down-regulation of Pw1, and also bi-allelic expression of the maternally expressed Zim2. A switch from maternally to bi-allelic expression of Zim2 could be explained if Pw1 and Zim2 share the same enhancers for their expression. If this is the case, Pw1 and Zim2 might compete in cis for these enhancers and down regulation of the transcriptionally dominant Pw1 might allow the expression of Zim2 in the paternal allele, resulting in the bi-allelic expression of Zim2. The paternal transmission of mutant allele also caused up regulation of Zim1, however the observed upregulation was still derived from maternal chromosome (Kim et al., 2012). Compared with paternal transmission, maternal transmission of the mutation had a mild impact on transcription and imprinting of Pw1 cluster genes, in that the levels of gene expression were almost unchanged in comparison with the wild type control. Interestingly, the maternal transmission of DMR mutation caused a switch in expression of Zfp264 from the paternal to maternal allele. One possible explanation could be that the maternal Pw1 DMR is active during one stage of development and the switch is consequence of deletion of Pw1 DMR on maternal chromosome. This is in accordance with the fact that homozygous mice are never born, although they should have the same expression pattern as a paternally transmitted deletion.

Although genetic work on mice gave us insight how the expression and imprinting of Pw1 gene cluster is regulated, we are still far from understanding the variety of phenotypes in mutant mice. How much of phenotypes can be attributed to the function of PW1 protein and how much comes from deregulation of expression of the genes from cluster is still unknown. Recently, Thiaville and colleagues (2013) confirmed that PW1 is a DNA binding zinc finger protein that acts as a transcription factor. Using chromatin immunoprecipitation with polyclonal anti-PW1 antibody they identified a PW1 DNA binding motif. PW1 binds to an AGTnnCnnnTGGCT consensus motif with its five zinc finger domains near C-terminus. They also identified direct
target genes for PW1 (Figure 6) and validated them by documenting their expression levels in the Pw1-DMR mutant mouse model with low levels of PW1 expression (Kim et al., 2012). Comparing the mutants with the wild type showed that the expression levels of targeted genes were reduced (Grb10, Mapk14, Nt5c1a, Ppargc1b, Setd1a, Syap1, Wnt9a), increased (Dusp1, Pgm2l1), or remained the same (Aebp2, Eml2, Ndufs8, Sdhb). These results indicate that PW1 could posses both repressor and activator function.

Direct targets of PW1 could explain a variety of phenotypes from different Pw1 mutant mouse lines. Metabolism and growth rate changes could be explained by PW1 directly regulating transcription of a subset of nuclear-encoded mitochondrial genes (Ndufs7, Ndufs8, Ppargc1b, Sdhb) and genes involved in modulating insulin/IGF-1 signalling (Mapk14, Dusp1, Grb10). Furthermore, PW1 alternation of maternal behaviour caused by defective neuronal connectivity (Li et al., 1999) could be connected to impaired Wnt signalling. Inhibition of the Wnt signalling pathway has been proposed to be necessary for specific tissue types (i.e. hypothalamus) to develop properly (Kapsimali et al., 2004). In this context observations of Wnt9a expression being influenced by PW1 binding need further investigation. Additional evidence of PW1 regulating Wnt signalling comes from work of Jiang and colleagues (2010). Enforced over-expression of Pw1 mRNA during zebrafish embryogenesis decreased β-catenin protein expression and inhibited Wnt-dependent tail development. Also the alternations in insulin/IGF-1 signalling pathways can have behaviour defects (Mitschelen et al., 2011; Devon et al., 2006), which makes identification of Dusp1, Mapk14, and Grb10 as direct PW1 targets intriguing.
Figure 6. Pw1 regulates a subset of genes through direct DNA binding

Chromatin immunoprecipitation was done with Pw1 antibody (A and B). A subset of genomic regions were demonstrated to be bound by Pw1 through both (A) regular PCR and (B) qPCR. (C) The expression levels of Pw1 bound genes were compared between wild type mice and mice with reduced Pw1 expression (DMR KO) by qRT-PCR. (Adapted from Thiaville et al., 2013)
**Pw1 marks adult stem cells in various tissues**

Like many other imprinted genes Pw1 is expressed at high levels in early embryonic mesoderm and is down regulated in tissues as they differentiate, remaining to be expressed in a small population of cells (Relaix et al., 1996). Pw1 is a part of imprinted gene network (IGN), which consist of a subset of imprinted genes that are down regulated postnatally, but in adulthood are predominantly highly expressed in somatic stem cells (Berg et al., 2011). A key feature of somatic stem cells is that they are generally considered quiescent, dividing infrequently, but are driven into cycle during periods of tissue regeneration or self-renewal. Berg and colleagues (2011) showed that members of the imprinted gene network are expressed in long-term hematopoietic cells (LT-HSC), satellite cells of skeletal muscle and epidermal stem cells of skin where they play a significant role in maintenance of “stemness”.

A small subset of IGN genes (Dlk1, Grb10, Gtl2, H19, Igf2, Mest, Ndn, Pw1, Plagl1) is expressed in majority of studied adult stem cell populations at higher levels compared to their differentiated progeny. To explore whether IGN gene expression correlates with functional stem cell properties Berg and colleagues (2011) stressed LT-HSC under two distinct conditions that mimic an acute response to injury (5-fluorouracil treatment) and chronic overstimulation (transplantation Irgm deficient bone marrow into irradiated mice). Thus, two conditions that disrupt LT-HSC homeostasis perturb the expression of a central group of imprinted genes known to be involved in embryonic and early postnatal growth.

Recently, Sassoon and colleagues using a Pw1-reporter mouse (Pw1nLacZ) showed that Pw1 identifies multiple adult stem cell populations in various tissues including skeletal muscle, intestine, testis, central nervous system, bone, bone marrow and skin (Besson et al., 2011) (Figure 7). In the same study, using an example of stem cells isolated from CNS, they showed that Pw1 expression is also a marker for stem cells in vitro. One of the characteristics of stem cells is their capability of self-renew and neural stem...
cells can be expanded in vitro by generating neurospheres. After first passage neurospheres contain mostly stem cells that express Pw1.

**Figure 7. Pw1:IRESnLacZ expression profile in various**

(A) Reporter activity and Pw1 expression identify progenitor cells in adult small intestine. Reporter activity and endogenous PW1 protein expression are restricted to the stem cell niche in basal crypt. (B) Co-localization of PW1 protein and LacZ in nestin positive neurospheres isolated from the reporter line after one passage. (C) Transverse sections of TA muscle from 7-week-old transgenic mice were stained for LacZ and Pw1 and did not reveal expression in myonuclei. (Adapted from Besson et al., 2011)
In skin PW1 protein and reporter activity were detected in the bulge, hair germ, and dermal papilla at all phases of the hair cycle and it was co-localized with known epidermal stem cell markers CD34, cytokeratins 5 and 15. Furthermore FACS sorted β-gal positive cells and transplanted into nude mice were capable of reconstituting hair follicles. When the grafts were challenged to regenerate, they demonstrated that only PW1 expressing cells were capable of robust regeneration. Lineage tracing with double labelled Pw1\(^{\text{nLacZ/H2bGFP}}\) cells revealed that PW1 positive cells also repopulate the hair follicle stem cell niches proving that PW1 marks the self-renewing stem-cell population in skin.

Pw1 as a marker of tissue resident stem cells was first described in postnatal skeletal muscle where PW1 expression is detected in satellite cells and in their precursors called PICs (PW1+/Pax7- interstitial cells) (Mitchell et al., 2010). Isolated from skeletal muscle PICs have bipotential behaviour \textit{in vitro} generating both smooth and skeletal muscle. Similar results were obtained by transplantation of PICs in injured skeletal muscle, where they gave rise to satellite cells and both smooth and skeletal muscle in comprising to transplantation of satellite cells that gave rise only to myofibres.

Using the transgenic reporter lines I identified Pw1 positive cells in the adult heart in significant numbers. Based on mRNA expression profiles and patterns during embryogenesis we surmise an epicardial origin. Immunohistochemistry and flow cytometric analysis revealed that Pw1 cells express the adult stem cell receptors Sca1, Pdgfra and Cd34 in similar manner like PICs do in skeletal muscle. Furthermore adult cardiac Pw1 cells are highly pro-angiogenic \textit{in vitro} and can induce tube formation when they are co-cultured with an endothelial cell line. Using pharmacological tools I locally depleted Pw1 cells which resulted in local cardiomyocyte atrophy and fibrosis. These results make Pw1 cells a potentially interesting component of regenerative medicine.
2. Materials and Methods

Animals

To detect Pw1 expressing cells we used two BAC transgenic reporter lines (Figure 8) Pw1\textsuperscript{nLacZ} (Besson et al., 2011) and Pw1\textsuperscript{eGFP} (Gong et al., 2003). In Pw1\textsuperscript{nLacZ} line IRES\textsuperscript{nLacZ} was introduced in 5' portion of Pw1 exon 9 and line was selected with single copy integration. The Pw1\textsuperscript{nLacZ} line was kept on a mixed C57Bl/6 and FVB background. For the Pw1\textsuperscript{eGFP} line the reporter gene was inserted immediately upstream of the coding sequence of the Pw1 gene, and the line was kept on mixed FVB and CD1 background.

Figure 8. Schematic representation of transgenes from PW1 reporter lines
Upper panel represents transgene from the Pw1\textsuperscript{nLacZ} line where IRES\textsuperscript{nLacZ}-pA cassette (blue) was introduced in exon 9 of Pw1 gene. Translation from mRNA from transgene results with nLacZ and non-functional truncated PW1 protein. Lower panel shows transgene from the Pw1\textsuperscript{eGFP} line where eGFP-pA (green) was introduced immediately after the Pw1 START codon resulting only in eGFP translation. Schematic representation was constructed according to descriptions from Besson et al., 2011 and Gong et al., 2003.
Genotyping was performed by PCR using genomic DNA from tail biopsies after over-night digestion (50 mM KCl, 10 mM Tris pH 8.0, 2 mM MgCl$_2$ 0.1 mg/ml gelatin, 0.45 % (v/v) NP-40, 0.45 % (v/v) Tween 20, 1 mg/ml proteinase K) at 56 °C. The PCR products were analyzed on 2 % agarose gels; primer sequence and PCR programs are provided below (Table 1). Depending on gel size, gels were run at the voltage between 100 and 120 V with constant current.

Table 1. Primer sequences and programs for genotyping of Pw1$^{nlacZ}$ and Pw1$^{egfp}$ transgenic reporter lines

<table>
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<tr>
<th>Mouse line</th>
<th>PCR primers (5’-3’)</th>
<th>PCR program</th>
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<tr>
<td>Pw1$^{nlacZ}$</td>
<td>ACCAACGTAACCTATCCCATT (sense)</td>
<td>94°C for 1 min</td>
</tr>
<tr>
<td></td>
<td>CTGATCTTCCAGATAACTGCC (anti-sense)</td>
<td>35 cycles</td>
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<tr>
<td></td>
<td></td>
<td>94°C for 1 min</td>
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<td></td>
<td></td>
<td>52°C for 30 s</td>
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<tr>
<td></td>
<td></td>
<td>72°C for 1 min</td>
</tr>
<tr>
<td>Pw1$^{egfp}$</td>
<td>GTGTGAGCAAAAACAGACAACCTGT GAAA (sense)</td>
<td>94°C for 5 min</td>
</tr>
<tr>
<td></td>
<td>TAGCGGCTGAAGCACTGCA (anti-sense)</td>
<td>10 cycles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94°C for 15 s</td>
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<tr>
<td></td>
<td></td>
<td>65°C for 30 s</td>
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<td>72°C for 40 s</td>
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<td>30 cycles</td>
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<td>94°C for 15 s</td>
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<td>55°C for 30 s</td>
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<td></td>
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<td>72°C for 40 s</td>
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Animals were housed in a clean, temperature controlled (22 °C) mouse facility on a 12-hour light/dark cycle. Mice were weaned around three weeks of age and housed in same sex groups of 3-5 per cage with pellet food and water ab libidum. All mouse procedures were approved by European Molecular Biology Laboratory Monterotondo Ethical Committee (Monterotondo, Italy) and were in accordance with national and European regulations.

For preparation of heart tissue and cells for histology and FACS, animals were anesthetised with avartin (0.5 mg/g) and perfused with HBSS solution without calcium supplemented with a 100 U/ml of heparin through the left ventrical. Tissues and cells for X-gal staining and FACS were processed as described below and for immunostaining mice were additionally perfused.
with 4 % (w/v) PFA in PBS, tissue was harvested and incubated in fresh 4 % (w/v) PFA in PBS over-night.

**Isolation of embryos**

To determine the development stages of the embryos, the days from the detection of the vaginal plug (E0.5) until time of dissection of the animals were counted. Dissections were always carried out around the same time of the day to minimize variation in developmental stages. Pregnant female animals were sacrificed by cervical dislocation and the uterus was isolated and washed with PBS. Embryos were then sequentially isolated from the uterus, the placenta and the extra-embryonic membranes. Embryos were sacrificed by cutting off the head and hearts were removed and processed separately for X-gal staining (E9.5, E10.5 and E11.5) or cell isolation (E11.5 and E12.5) or whole embryos were processed (E9.5).

**Local inactivation of Pw1 cells using Daun02**

The “Daun02 inactivation method” is an approach for selective inactivation of LacZ positive cells, previously described on nucleus accumbens neurons in rats (Koya et al., 2009) (Figure 9). Daun02 was administrated into the pericardial sac of Pw1<sup>nLacZ</sup> mice in the following way.

Animals were anesthetized using 2 % isoflurane and endo-tracheal intubation was carried out to ventilate the lungs while the chest was open. An incision of several mm length away from the sternal border towards the right armpit was made. Underlying connective tissue was opened and the two muscle layers underneath were spread and held by retractors without incision into the muscle. The rib cage was opened at 4<sup>th</sup> intercostal space to allow access to the heart without damaging the pericardial sac. In total 50 µg of Daun02 in 500 µl of 2 % (w/v) sodium alginate was administrated into the pericardial sac. For the sham operated animals only 500 µl of 2 % (w/v) sodium alginate was administrated. The retractors were removed and the chest was closed by bringing together the 4<sup>th</sup> and 5<sup>th</sup> ribs using 6-0 nylon
sutures. The muscles were placed into the original position and the skin was closed using 6-0 nylon sutures. Mice were monitored until awakening and body temperature was kept up by heat pad. Mice were sacrificed 7 days post surgery.

**Figure 9. Schematic mechanism for Daun02 inactivation in Pw1
\textsuperscript{nLacZ} mouse heart**

The Pw1\textsuperscript{nLacZ} transgene contains a Pw1 promotor that drives expression of LacZ, encoding β-galactosidase (β-gal). β-galactosidase can catalyze the conversion of the prodrug Daun02 into daunorubicin which reduces polymerase activity affecting regulation of gene expression (adapted from Koya et al., 2009).
Tissue processing and histochemistry

X-gal staining

After perfusion tissues were excised and prepared for X-gal staining in the following way. For whole-mount staining total hearts were fixed in ice-cold X-gal fix buffer (0.1 M phosphate buffer pH 7.3, 5 mM EGTA pH 7.3, 2 mM MgCl2 and 0.2 % (w/v) glutaraldehyde) for 30 min on ice. Following the fixation tissues were washed three times for 20 min with 0.1 M phosphate buffer (pH 7.3) supplemented with 2 mM MgCl2, 0.02 % (v/v) NP-40 and 0.01 % (w/v) sodium deoxycholate and incubated in X-gal staining solution (washing buffer supplemented with 5 mM K4Fe(CN)6, 5 mM (K3Fe(CN)6 and 1 mg/ml X-gal). For thin sections hearts after perfusion were embedded in OCT and immediately frozen on dry ice. Ten-micron thick tissue sections were cut and air dried for 2-3 min and stored at -80 °C until staining. Frozen sections were fixed for 10 min in X-gal fix buffer and washed twice for 5 min in X-gal wash buffer. Sections were placed in X-gal staining solution and incubated at 37 °C for 4 h. Whole-mount tissue and frozen sections were post-fixed with 4 % (w/v) PFA in PBS and frozen sections were counterstained using eosin.

Immunohistochemistry

Heart tissue after overnight post-fixation was washed in PBS and embedded in 3 % (w/v) low melting agarose. For 100 µm thick section staining, tissue was sectioned using a vibrating blade microtome (Leica Microsystems). The thick sections were permeabilised and blocked with blocking buffer (1 % (v/v) normal goat serum, 0.5 % (v/v) Triton X-100, 0.5 % (w/v) BSA, 0.1 % (w/v) glycine in PBS) for 2h with light agitation. After permeabilisation and blocking floating sections were incubated overnight in primary antibodies (Table 2) and following day washed three times for 30 min with blocking buffer and then incubated with secondary antibodies (Table 2) for 3h. Next sections were washed two times with blocking buffer and the
nuclei were contra-stained with DAPI in PBS supplemented with 0.5 % (v/v) Triton X-100 and 0.1 % (w/v) glycine for 30 min. After nuclear staining with DAPI sections were washed three times for 30 min in PBS with 0.1 % (w/v) glycine and mounted on glass slide. Fluorescent microscopy images were obtained using Leica SP5 confocal laser scanning microscope (Leica Microsystems). Two and three-dimensional images were prepared using Imaris software (Bitplane), with adjustments made to brightness and contrast.

Table 2 List of primary and secondary antibodies and their dilution used for immunohistochemistry.

<table>
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<tr>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-LacZ (Capel)</td>
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<td>Streptavidin Alexa 647 (Invitrogen)</td>
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Ultracryotomy, immunogold labelling and electron microscopy

After perfusion tissue samples were post-fixed with 4 % (w/v) PFA in 0.1 M sodium phosphate buffer, pH 7.1. Samples were cut into 0.5 mm3 squares, embedded in 12 % (w/v) gelatine and infused in 2.3 M sucrose (Painter et al., 1973; Tokuyasu et al., 1973). Mounted gelatine blocks were frozen in liquid nitrogen and ultrathin (50nm) cryosections were cut at -120˚C with an Ultracryo-microtome (Leica). Sections were retrieved in 1.15 M sucrose in 2 % (v/v) methylcellulose solution and processed for immunolabelling. After blocking step with 0.5 % (w/v) BSA, single immunolabelling was performed in a humid chamber with primary antibody (rabbit anti-LacZ, Capel, 1:25) and protein A coupled to 10 nm diameter gold particles, PAG-10nm (CMC, University Medical Center, Utrecht, The Netherlands).

Haematoxilin/eosin (H&E) staining

Frozen sections were fixed with 4% (w/v) PFA in PBS for 15 min and washed three times with PBS for 10 min. Slides were rinsed in distilled water and incubated in Harris haematoxilin solution for 1 min. Slides were washed in distilled water, treated with acidic alcohol (1% HCl, 70% ethanol) for 30 s, washed again with distilled water and incubated with 1% eosin solution for 1 min. After washing for 30 s with distilled water, slides were incubated in 95% ethanol (30 s), 100% ethanol (2 min) and xylene (3 min) before mounted with Eukitt quick hardening mounting medium.
Preparation of single cell suspensions from heart and flow cytometric analysis and sorting

After perfusion with HBSS supplemented with 100 U/ml of heparin hearts were finely minced with surgical scissors and incubated in 10 ml of 2 mg/ml collagenase type II/HBSS (with calcium and magnesium) enzyme solution for 60 min at 37 °C with gentle agitation. Dissociated heart tissue was filtered through 70 µm filters, mixed with 5 ml of FBS and centrifuged at 250×g for 5 min. Cells were processed for staining for FACS. LacZ positive cells were detected with a fluorogenic substrate, FDG, of galactosidase (LacZ enzyme) (Nolan et al., 1988; Guo and Wu, 2008).

Cells from Pw1nLacZ hearts were resuspended in 100 µl of HBSS and incubated at 37 °C for 10 min. Next 100 µl of pre-warm FDG solution (2 mM FDG in water) was added to the cell suspension and incubated further at 37°C for exactly 1 min. The FDG loading into the cells was stopped with 10 ml of ice-cold FACS staining buffer (HBSS, 2% FBS, 10 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin) and cells were incubated in the dark for 90 min on ice. Next cells were centrifuged and proceed with standard FACS staining protocol.

Cells were re-suspended into FC receptor blocking solution consisting of FACS staining buffer and Cd16/Cd32 (eBioscience) (1:50) and incubated for 10 min. Next fluorophore coupled antibodies for FACS were added as described in the table (Table 3) and incubated for 10 min (for Cd34 staining cells was incubated for 60 min). Cells were centrifuged and washed with FACS staining buffer and re-suspended in the same buffer supplemented with living die Sytox Blue or 7AAD to exclude the dead cells. Fluorescence activated cell sorting and analysing was conducted using FACS-ARIA cell sorter (Becton Dickinson) and the using FlowJo Software.
Table 3 List of antibodies and their dilution used for FACS staining.

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<tr>
<td>Cd34 APC (eBioscience)</td>
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<td>Cd117 APC (eBioscience)</td>
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Transcriptome analysis

Isolation of RNA and quantitative real time PCR

For total RNA from the heart snap frozen ventricles were homogenized in ice-cold TRIzol (tissue : TRIzol = 20 mg : 500 µl) and 20 mg of tissue homogenate was used for RNA extraction. TRIzol was also used in total RNA extraction from FACSsorted Pw1 cells. Cells were sorted in 500 µl of TRIzol and vigorously vortexed to dissociate cells. Next 100 µl of chloroform was added to 500 µl of tissue /cell homogenate and after 2-3 min of incubation on room temperature homogenate was centrifuged at 12 000 × g, 4 °C for 15 min. The aqueous phase after centrifugation was separated in the new tube and mixed with 70 % ethanol in ratio 1:1. Then RNA was cleaned using RNeasy Mini Kit (QIAGEN) and the RNA concentration was determined with spectrophotometer. After RNA quality verification, 1-2 µg of RNA was used for DNaseI treatment and cDNA synthesis using QuantiTect Reverse Transcription Kit (QIAGEN). TaqMan gene expression assays (Applied Biosystems) were used for relative quantification of the mRNA levels of the genes of interest. Probes for TaqMan Gene Expression Assays are as listed in the table below (Table 4).

Expression levels of genes of interest were analysed by normalizing against expression levels of house keeping gene Gapdh using the ΔΔC(T) method. To transform Ct values to linear expression values, the following formula was used:

Relative expression = 2^(-ΔCt-Ct (stable))

ΔCt =Ct (gene) – Ct (Gapdh)

Ct (stable): Ct one constant sample across all Cts for the same gene

Statistical analysis was performed using the Student’s t test (tail 2, type2). Values were expressed as mean ± standard error and differences with p value < 0.05 were considered significant.
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</table>
GeneChip analysis

Expression analyses were conducted on FACS sorted Pw1<sup>EGFP</sup><sub>N/L</sub> and Pw1<sup>EGFP</sup><sub>H</sub> cells from the mouse heart and compared with the RNA isolated from the total mouse heart. Isolation of RNA was done as described above and the RNA quality was analysed with a Bioanalyzer (Agilent Technologies) and further processed at Genecore (EMBL Heidelberg, Germany).

Microarrays experiments were performed on Affymetrix GeneChip MoGene-2.0 Array. Data were extracted and analyzed on GeneSpring GX 12.5 software. All gene array raw data was first normalized in order to make multiple chip data comparable and then filtered on their signal intensity values. The range of intensity values to pass the filter was set between 20 and 50 percentile. Furthermore a list of Pw1 cell-enriched genes was created by using parametric unpaired t test with unequal variance. Of the resulting genes, Pw1 cell genes were defined as genes with a normalized >2-fold increased expression compared to the total heart expression. After normalization to total heart expression created gene lists of Pw1 cell gene populations were compared between each other.

To determine the categories of genes upregulated in Pw1 cells, we tested whether any Gene Ontology (GO) terms were overrepresented for the gens found to be up-regulated compared with the total heart samples. Using the publicly available software GeneCodis14 28 GO terms (Table S 2) were found to be significantly enriched in this set of upregulated genes (Hypergeometric test16 p-value<0.05, FDR17 adjusted). Furthermore a KEGG and Panther pathway enrichment analysis was performed using GeneCodis14 and revealed 10 enriched pathways.
Primary cell cultures

Cultures of adult Pw1 cells and transplantation assays

For Pw1 cultures cells were FACS sorted into Dulbecco’s modified Eagle’s medium (DMEM) containing 10 mM HEPES, 1mM sodium pyrovate, 2 mM L-glutamine, 1 × nonessential amino acids, 15% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. After the sorting Pw1 cells were plated on collagen type I coated chamber slides and incubated at 37 °C, 5% CO₂ for 8 days. Media was changed every 4 days. Cells were fixed with 4% PFA and stained for phalloidin and LacZ.

The matrigel plug assay was used as in vivo angiogenesis model. Briefly, after FACS sorting Pw1 cardiac cells with or without endothelial cells were resuspended in 200 µl of matrigel on ice and was implanted into Rag-/- mice by subcutaneous injections. Mice were sacrificed after 8 days from implantation.

Embryonic Pw1 cardiac cultures

Embryonic epicardial derived cells were isolated and cultured as previously described with some modifications (Smart and al., 2007; Chen et al., 2002). Embryonic hearts from the development stage E11.5 and E12.5 were dissected and cleaned from the extra tissue. The whole hearts were plated onto collagen type I coated 12 well dishes in a single drop of M199 media supplemented with 100 ng/ml Tβ4. Plates were placed in humidified chamber and incubated over-night in cell incubator at 37 °C, 5% CO₂. Next day a 500 µl of fresh media with Tβ4 was added and hearts were incubated for additional 24h. Epicardial cells migrated away and formed a monolayer surrounding the remaining cardiac tissue, which was removed using the forceps. Epicardial cells were further cultured in M199 media supplemented with 15% of FBS.
Biochemistry

To investigate the role of Pw1 in growth factor signalling mouse Pw1 cDNA was cloned into pcDNA3.1(-) vector, designed for high-level constitutive expression in mammalian cells. For inducible expression of Pw1 stable NIH 3T3 cell line was made by introducing mouse Pw1 cDNA into Tet-On pTRE3G plasmid and transfecting NIH 3T3 Tet-On cells. For detecting the Pw1 HA tag with the Kozak consensus sequence was added before the first AUG (Pw1 start of translation) site (Figure 10).

Figure 10. Schematic representation of CMV:Pw1 plasmid for transient and pTRE3G:Pw1 plasmid for inducible stable over-expression of Pw1
The Pw1 cDNA was cloned under CMV promotor into pcDNA3.1(-) plasmid (left panel) or into Tet-On pTRE3G plasmid (right panel). To track PW1 protein expression HA tag and Kozak sequence were cloned in front of START codon for Pw1 translation.
Transformation of bacteria for plasmid amplification

One Shot TOPO10 competent E. coli was used for plasmid amplification. Cells were thawed on ice and 5 µl of plasmid was added. Following 5 min of incubation cells were heat-shocked for 30 s at 42 °C and placed on ice for 2 min. Immediately after, 50 µl of transformed cells were seeded on pre-warmed selective plate containing 100 µg/ml ampicillin and incubated over-night. Next day individual colonies were picked and cells containing the plasmid were grown 24h at 37 °C in 200 ml of LB medium supplemented with 100 µg/ml ampicillin. Cells were centrifuged at 8000 × g for 5 min and plasmid was purified following the plasmid purification kit (QIAfilter Plasmid Purification Kit, Qiagen). The quality and quantity of purified plasmid was assessed spectrophotometrically by measuring absorption at wavelengths of 260 nm and 280 nm. The right cloning was confirmed by DNA sequencing.

Culture conditions of NIH 3T3 and HEK 293 cell lines

Human HEK 293 epithelial cells were maintained on plastic tissue culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 10 mM HEPES, 1mM sodium pyrovate, 2 mM L-glutamine, 1 × nonessential amino acids, 10 % (v/v) heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. The embryonic fibroblast cell line NIH 3T3 were cultured in DMEM supplemented with 2 mM L-glutamine, 1 × nonessential amino acids, 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin.

Transient transfections of HEK 293 cells were done using Xfect transfection reagent (Clonetech) with some modifications. Cells were grown on 12-well plates in 500 µl of growth media and at the 80 % of confluence cells were transfected with 2.5 µg of CMV:Pw1 or mock plasmid DNA. First, plasmid DNA was mixed in 50 µl Xfect Reaction Buffer and than 0.75 µl Xfect polymer was added. The transfection mixture was vigorously mixed and
incubated at room temperature for 10 min. The transfection mixture was added dropwise to the cells. After 48h cells were harvested for protein or immunohistochemistry.

For making stable inducible Pw1 expression, Tet-On NIH 3T3 cell line was transfected by electroporation with pTRE3G:Pw1 plasmid. Cells were harvested at the 80 % of confluence and prepared for electroporation according to Amaxa protocol. Four million cells were resuspended in 100 µl of electroporation buffer (7.12 mM ATPNa₂, 11.6 mM MgCl₂, 144 mM KH₂PO₄, 23.3 mM NaHCO₃, 3.63 mM glucose) with 5 µg of pTRE3G:Pw1 plasmid. After applying electrical pulse cells were incubated for 10 min in pre-warmed RPMI medium and plated afterwards in complete NIH 3T3 medium described before. After three passages, Pw1 overexpression was induced by adding doxycycline to the cells at final concentration of 1 µg/ml. For RNA analysis, after 24 and 48h of induction cells were trypsinized and FACS sorted based on RFP expression. Total RNA was extracted as described before.

**Preparation of protein lysates from cultured cells and western blot analysis**

Cultured HEK 293 cells were washed two times with DMEM and collected into cell lysis buffer (50 mM Tris pH 7.0, 150 mM NaCl, 5 mM NaF, 2 mM NaVO₃, 50 mM β-glycerophosphate, 1 % (v/v) NP-40, 0.25 % (w/v) Na-deoxycholate, 1 mM PMSF, 1 mM EDTA pH 7.5 and complete proteinase inhibitors (Roche)). Lysates were sonicated for 20-30 s and centrifuged for 10 min at 10 000 × g and 4 °C. Protein concentration of supernatant was determent by a dye-binding technique (Bradford assay and protein extracts were stored at -80 °C.

For SDS PAGE protein extracts were diluted in Laemmli sample buffer (32 % (v/v) glycerol, 4 % (w/v) SDS, 125 mM Tris pH 6.9, 0.1 % (v/v) β-mercaptoethanol, 0.01 % (w/v) bromphenol blue) and denaturated for 5 min at 95 °C. Approximately equal amounts of proteins (20 µg per well) were loaded on 8 % (w/v) polyacrylamide gels with SDS (Biorad) and electrophoresis was performed at constant voltage (200 V for 1h) and room temperature.
Following SDS PAGE, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane, which has been activated in methanol and equilibrated in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 10 % (v/v) methanol). Transfer was carried out in a wet transfer apparatus for 1.5 hours at constant voltage (100 V) and 4 °C. After the transfer proteins on the membrane were stained and fixed with Rouge Ponceau S. After the washing in TBS buffer (20 mM Tris pH 7.5, 500 mM NaCl) the membranes were blocked in 5 % (w/v) BSA in TBST buffer (20 mM Tris pH 7.5, 500 mM NaCl, 0.1 % (v/v) Tween 20) for 1 h at room temperature. Primary antibodies were diluted in blocking buffer (table) and incubated over-night at 4 °C. Next day membranes were washed three times for 5 min in TBST buffer and incubated with secondary antibodies in blocking buffer for 1 h at room temperature. Following three times wash in TBST buffer specific bands were detected with chemiluminescence.

Table 5 List of antibodies and their dilution used for Western staining

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<tr>
<th>Antibody</th>
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<tr>
<td>HA-tag (Santa Cruz)</td>
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<tr>
<td>PW1 (provided by Professor David Sassoon from UPMC Paris VI)</td>
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</tr>
<tr>
<td>α-tubulin (Sigma)</td>
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<td>ECL Anti-rabbit IgG (Amersham)</td>
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<tr>
<td>ECL Anti-mouse IgG (Amersham)</td>
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</table>
3. Results

Pw1 is expressed in the adult steady state heart

For describing Pw1 expression patterns in the adult steady state hearts I used Pw1\textsubscript{nLacZ} reporter mouse line (Besson et al., 2011). Eight to twelve weeks old mouse hearts or 10 µm thin cryo-sections were stained for β-galactosidase activity (Figure 11). Whole mount staining revealed high expression levels of the reporter in atrium and on the surface of the ventricles. Cross-sections of the heart showed abundance of interstitial and epicardial cells positive for LacZ. Expression of the reporter was limited to a subset of cells within the epicardium. This is in the line with recent studies showing that the epicardium, and also the proepicardial organ, is a not homogeneous cell population during development, but rather a very heterogeneous one (Bochmann et al., 2010; Katz et al., 2012).

![Figure 11. Pw1\textsubscript{nLacZ} expression profile in adult mouse heart](image)

Hearts of 8-12 weeks old animals were stained for β-galactosidase activity. (A) Whole mount staining show different expression levels of nLacZ between atrium and
ventricle. (B) On cross-sections one can nicely see distribution of Pw1 cells in adult myocardium and (C) at higher magnification the heterogeneous localization of Pw1 cells in epicardium.

**Flow cytometric profiling of Pw1 ventricular cells**

As Pw1 was previously described as a potential adult stem cell marker I analysed Pw1 expressing cells from an nLacZ reporter line for known cardiac stem cell receptors (Figure 12). To detect Pw1 cells I used FACS-based detection of LacZ positive cells with a well described fluorogenic β-galactosidase substrate FDG. Galactosidase cleaves FDG and releases a fluorescence product FITC. Using a combination of FACS-based LacZ detection and antibody staining for receptors I found that a subset of Sca1, Pdgfra, Cd34 and Cd29 cells are also expressing Pw1. There is not a 100% overlap between any of the markers and Pw1. Interestingly Pw1 cells do not express c-Kit, previously described as a bone marrow derived cardiac stem cell marker, but the majority of Pw1 cells express Sca1, Cd29 and Pdgfra, which were described as receptors of epicardium-derived cells (EPDCs).
**Figure 12. Immuno-phenotype of Pw1 ventricular cells from adult heart**

(A) Representative FACS scatter profiles showing Sca1, Pdgfra, Cd34 and Cd29 heart populations from wild type and Pw1:nLacZ reporter mice. The gates show a subpopulation of stem cell receptor positive cells (Sca1, Pdgfra, Cd34 or Cd29) that are also LacZ positive. Blood and endothelial cells were excluded based on Cd45 and Cd31 expression. (B) Quantification of the staining for stem cell receptor (Sca1, Pdgfra, Cd34, Cd29 or c-Kit) and Pw1 (LacZ). Values are presented as the mean percentage ± SEM, n=4.
Gene expression of Pw1 cells based on Sca1 expression

To further investigate the origin of the Pw1 cells I sorted two populations based on Sca1 expression (Sca1 low/negative and Sca1 high population) and compared expression levels of Pw1, epicardial and EMT markers to the expression levels from total heart extract (Figure 13). Interestingly Pw1 RNA levels were differently expressed between Sca1 negative/low (Sca1\text{NL}) and Sca1 high (Sca1\text{H}) population based on gender of the mice. The expression levels of adult epicardial markers such as Upk3b, Wt1 and Id1 were higher in Sca1\text{NL} than in Sca1\text{H} population. Both populations were enriched in Raldh2, Tbx18 and Tcf21 (markers of epicardial activation) in comparison to RNA levels from total heart extract. Both populations were also enriched in EMT inducers Snail, Slug and Twist1 which suggests an epicardial/proepicardial origin of the Pw1 cells.
Figure 13. Gene expression profile of Pw1 cells based on Sca1 expression

(A) Flow cytometric sorting of single cells based on LacZ (Pw1) and Sca1 expression from the adult heart. The gates used to sort Pw1\textsuperscript{LacZ}\textsuperscript{Sca1\textsuperscript{NL}} and Pw1\textsuperscript{LacZ}\textsuperscript{Sca1\textsuperscript{H}} populations are shown in red and blue. Blood and endothelial cells were excluded based on Cd45 and Cd31 expression. (B) Pw1 expression levels by qRT-PCR on
sorted Pw1 populations from male and female animals are normalized to total heart expression (H). Histograms show mean ± SEM, n=3. (C) qRT-PCR analysis of selected epicardial (Upk3b, Wt1 and Id1), epicardium activated (Raldh2, Tbx18 and Tcf21) and EMT (Snail, Slug and Twist1) genes on isolated Pw1 populations. Values are presented as the relative expression normalized to the expression of total heart extract ± SEM, n=6.

**Pw1 heart cells during development**

To determine the developmental time course for Pw1 expression I used Pw1<sup>αLacZ</sup> mouse reporter line. The Pw1 gene is expressed in majority of the tissues during early embryonic development and at later stages becomes restricted to small number of cells. At the age of E9.5 of mouse embryonic development Pw1 reporter activity can be detected in majority of the cells except in the heart (Figure 14a and 14b). At later stages (E10.5 and E11.5) heart contains abundant Pw1 cells (Figure 14c and 14d). This expression pattern co-responds with the formation of epicardium from proepicardial organ. At E9.5 proepicardial cells migrate towards the heart tube. As they reach the heart they form the epicardium and migrate over the heart. The migration follows well-defined pattern. As the cells reach the heart they cover the dorsal surface of ventricle and continue towards the atrium and subsequently cover the ventral surface of the ventricle. The cells then cover the outflow tract and atria and by E11.5 the entire heart is engulfed by the epicardium. This timing perfectly follows the envelopment of developing heart by epicardial layer.
Figure 14. Pw1 expression patterns during early heart development
Whole-mount β-galactosidase (X-gal) staining of (A) E9.5 embryo and (B) E9.5, (C) E10.5 and (D) E11.5 embryonic heart. Reporter activity is detected in the embryonic heart from the stage E10.5 and continues to be expressed during adulthood. This pattern of expression correlates with cell invasion from the proepicardial organ and formation of epicardium.
The Pw1 transcription profile

To investigate the role of Pw1 cells I analyzed their gene profile in normal steady state heart. Using Pw1\textsuperscript{eGFP} reporter line Pw1 cells were isolated based on eGFP and Sca1 expression and total RNA was isolated as described in Materials and Methods. RNA was analyzed by exon expression microarray and compared to total RNA isolated from ventricle tissue. To confirm that expression of eGFP is overlapping with expression of nLacZ reporter I performed immuno-staining on double transgenic Pw1:nLacZ/Pw1:eGFP mice. The staining for nLacZ and eGFP showed a 100% overlap (Figure 15).

**Figure 15.** Expression of eGFP and nLacZ in double transgenic mouse Pw1\textsuperscript{eGFP/nLacZ} is overlapping in cardiac Pw1 cells

Immunofluorescent staining of cardiac Pw1 cells from double transgenic Pw1\textsuperscript{eGFP/nLacZ} reporter show overlapping expression of eGFP and nLacZ.
In order to compare differentially expressed genes between Pw1eGFP Sca1\textsuperscript{NL} and Pw1eGFP Sca1\textsuperscript{H} populations, individual populations were normalized with the Gapdh expression from ventricle tissue. Analysis focused only on genes enriched in Pw1 cells compared with total ventricles (Table 6 and Table 7) and revealed 795 up-regulated genes in Pw1eGFP Sca1\textsuperscript{NL} and 729 up-regulated genes in Pw1eGFP Sca1\textsuperscript{H} population. Comparison of enriched genes between the two populations after normalization to total ventricle tissue showed 467 commonly expressed genes, 328 genes specific for Pw1eGFP Sca1\textsuperscript{NL} and 729 genes specific for Pw1eGFP Sca1\textsuperscript{H} population (Figure 16A). In both populations we found up-regulated previously described epicardial and EMT genes (Figure 16B).

To determine the categories of genes up-regulated in Pw1 populations we performed Gene Ontology (GO) Biological Process, KEGG and Panther pathways analysis (Hypergeometric test 16 p-value<0.05, FDR17 adjusted) using publicly available software GeneCodis14 (Figure 17). We found the same categories of genes up regulated in both Pw1eGFP Sca1\textsuperscript{NL} and Pw1eGFP Sca1\textsuperscript{H} populations. The majority of gene categories include extracellular matrix remodelling, focal adhesion, angiogenesis, TGF\textbeta, MAPK and integrin signalling pathways. Overall, enriched genes revealed modest differences between the two populations of Pw1 cells, mainly in extent of induction.
Figure 16. Microarray gene expression analysis of Pw1 populations

(A) Venn diagram of differentially expressed genes from Pw1\textsuperscript{eGFP}\textsuperscript{Sca1\textsuperscript{NL}} and Pw1\textsuperscript{eGFP}\textsuperscript{Sca1\textsuperscript{H}} cells. Analysis reveals 467 commonly expressed genes, 328 genes specific for Pw1\textsuperscript{eGFP}\textsuperscript{Sca1\textsuperscript{NL}} and 729 genes specific for Pw1\textsuperscript{eGFP}\textsuperscript{Sca1\textsuperscript{H}} population (B) Histograms of fold enrichment for the up-regulated epicardial and EMT genes in Pw1 populations over total ventricle tissue is presented and the cut off value is 2.0.
Table 6. List of enriched genes in Pw1^eGFP Sca1^N/L population

List of top 20 genes expressed in Pw1^eGFP Sca1^N/L heart population. Fold enrichment of the genes expressed in Pw1^eGFP Sca1^N/L population over total heart is presented and the cutoff value is 2.0.

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<td>Il6</td>
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<td>19.157991</td>
<td>Fbn1</td>
<td>fibrillin 1</td>
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<td>Sema3c</td>
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<td>15.60975</td>
<td>Hspa1a</td>
<td>Hspa1b</td>
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Table 7. List of enriched genes in Pw1\textsuperscript{eGFP}Sca1\textsuperscript{H} population

List of top 20 genes expressed in Pw1\textsuperscript{eGFP}Sca1\textsuperscript{H} heart population. Fold enrichment of the genes expressed in Pw1\textsuperscript{eGFP}Sca1\textsuperscript{H} population over total heart is presented and the cut off value is 2.0.

<table>
<thead>
<tr>
<th>Fold of enrichment</th>
<th>Target ID</th>
<th>Definition</th>
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<tr>
<td>106.60513</td>
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<tr>
<td>69.37048</td>
<td>Hspa1b</td>
<td>heat shock protein 1B</td>
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<td>57.1611</td>
<td>Cxcl1</td>
<td>chemokine (C-X-C motif) ligand 1</td>
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<td>Fosb</td>
<td>FBJ osteosarcoma oncogene B</td>
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<td>38.029053</td>
<td>Fos</td>
<td>FBJ osteosarcoma oncogene</td>
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<td>activating transcription factor 3</td>
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<td>19.648472</td>
<td>Nop58</td>
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<td>Pdgfra</td>
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<td>protein phosphatase 1, regulatory (inhibitor) subunit 15A</td>
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<td>B cell translocation gene 2, anti-proliferative</td>
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<td>13.80541</td>
<td>Socs3</td>
<td>suppressor of cytokine signaling 3</td>
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Figure 17. Gene Ontology, KEGG and Panther pathway analysis of Pw1 signature genes
GO Biological process, KEGG and Panther pathway analysis of Pw1\textsuperscript{eGFP}\textsuperscript{Sca1\textsuperscript{N/L}} (red) and Pw1\textsuperscript{eGFP}\textsuperscript{Sca1\textsuperscript{H}} (blue) cells show enrichment of genes involved in extracellular matrix remodelling, angiogenesis and signalling pathways implicated in cell migration and EMT (TGFβ, MAPK, PDGF and integrin signalling pathways).

**Cardiac Pw1 cells are associated with the capillary network**

As the GO ontology analysis of genes specific for the Pw1 cells suggested the role in extracellular matrix remodelling and blood vessel development and angiogenesis, I looked more closely into the relationship between endothelial and Pw1 cells. Pw1\textsuperscript{eGFP} transgenic heart sections were co-stained with capillary marker isolectin B4 (IB4) (Figure 18a) and the images were obtained using a confocal laser scanning microscope. Results showed that the Pw1 cells form 3D networks closely associated with cardiac capillaries. The same results were obtained on Pw1\textsuperscript{nLacZ} reporter line with cryo-immuno electron microscopy (Figure 18b). Using secondary antibodies coupled with gold particles I observed Pw1 cells, with specific morphology, in near proximity of endothelial cells.

Gene expression profile from FACS sorted Pw1 cells from Pw1\textsuperscript{nLacZ} reporter revealed that Pw1 cells secrete numerous growth factors involved in promoting angiogenesis (Figure 19). Growth factors such as angiopoietins (Angpt1 and Angpt2), insulin like growth factors (Igf1 and Igf2), Vegfa, Jag1, thymosin β4 and growth factors from TGFβ family are required for angiogenesis during development and maintenance of mature blood vessels during adulthood. The qRT-PCR results also showed differences in growth factor expression between the two Pw1 populations suggesting heterogeneity between the two.

To investigate relationship between endothelial and Pw1 cells I used a matrigel plug assay as a model for in vivo angiogenesis (Figure 20). I isolated Pw1\textsuperscript{eGFP} cells from CAB:dsRed mice and injected them with endothelial cell line (bEND.3) in matrigel under the skin of immunodeficient Rag-/- mice. After seven days mice were sacrificed and matrigel plugs were analysed. Immunofluorescent staining for endothelial marker VE-Cad and dsRed (Pw1
cells) revealed close proximity of endothelial cell and Pw1 cell networks (Figure 20a). The H&E staining showed presence of structures that indicate endothelial tube formation when bEND3. cells were cultured with Pw1 cells (Figure 20b).
Figure 18. Heart Pw1 cells are associated with the cardiac capillary network
(A) Max projections of Pw1:eGFP cells with perivascular position in myocardium with longitudinal (left) and transversal (right) cardiomyocyte orientation (capillaries stained...
with IB4 and nuclei with DAPI). (B) Electron microscopy image of perivascular nLacZ (+) Pw1 cells from the adult myocardium.

![Graphs showing expression levels of various growth factors in Pw1 cells](image)

**Figure 19. Enriched expression of growth factors in Pw1 cells**

Expression of angiogenic factors was measured by qRT-PCR in sorted Pw1 populations (Sca1 L/N and Sca1 H) from Pw1:nLacZ hearts. Expression values are presented as the relative expression normalized to the expression of total heart extract ± SEM, n=6.
Figure 20. Matrigel plug assay using bEND.3 with Pw1/dsRED cells

The matrigel plug assay was used as *in vivo* angiogenesis model. FACS sorted Pw1<sup>EGFP</sup> cells from CAB:dsRed reporter mice were injected subcutaneously with bEND.3 cells (n=3). (A) Immunofluorescent staining for dsRed and VE-Cad and (B) H&E staining of matrigel gels shows close relationship between endothelial and Pw1 cells. Arrows indicate structures resembling endothelial tubes.
Pw1 cells during physiological heart hypertrophy caused by pregnancy

To investigate the role of Pw1 cells in adult cardiac remodelling I investigated the extent and patterns of Pw1 gene activation in pregnancy-induced cardiac hypertrophy (Figures 21 and 22). During pregnancy, maternal hearts undergo physiological hypertrophy and show increases in size and mass of cardiomyocytes. Measuring total heart mass and normalizing it to tibia length, I confirmed that the majority of heart growth happens already during the first week of pregnancy (Figure 21b), with heart growth preceding the body mass increase during pregnancy. Whole-mount X-gal staining reveals activation of epicardial Pw1 cells. Similar to non-pregnant hearts, epicardial Pw1 cells on the surface of pregnant hearts (7.5 days) show distinct patterns, with wider stripes consisting of increased Pw1 cells. Immunohistochemistry revealed the same up-regulation of Pw1 cells in interstitium of pregnant hearts (Figure 21d).
Figure 21. Physiological cardiac hypertrophy during pregnancy is accompanied with increased numbers of Pw1 cells

(A) Comparison of non-pregnant and seven days pregnant hearts. (B) According to the heart weight to tibia length measurements, the majority of cardiac growth happens in first week of pregnancy preceding the body weight increase. Values are presented as the mean ± SEM, n=6 (C) Whole mount β-galactosidase (X-gal) staining shows increased numbers of Pw1 cells on the surface of the heart in pregnant animals. Immunofluorescent staining (D) for Pw1 reporters also showed higher numbers of Pw1 cells in interstitium of cardiac muscle.
To investigate up-regulated genes and pathways in cardiac Pw1 expressing cells during physiological cardiac growth I compiled a list of common genes between Pw1$^{eGFP}$Sca1$^{N/L}$ and Pw1$^{eGFP}$Sca1$^{H}$ populations from non-pregnant hearts after normalization to total ventricular tissue and compared it to the list of Pw1 specific genes from 7.5 days pregnant hearts (Figure 22a). Comparison of enriched genes from non-pregnant and pregnant Pw1 cells showed 358 commonly expressed genes, 109 genes specific for Pw1 cells from un-pregnant and 251 genes specific for Pw1 cells from pregnant hearts.

To determine the categories of genes up regulated in the Pw1 population during pregnancy I performed KEGG and Panther pathways analysis (Hypergeometric test 16 p-value<0.05, FDR adjusted) using publicly available software GeneCodis14 (Figure 22b). Major gene categories include pathways involved in protein processing in endoplasmic reticulum, regulation of inflammation and pathways previously described as important in EMT and pregnancy induced heart hypertrophy such as TGFβ, Wnt, MAPK, PI3 kinase and focal adhesion pathways (Chung et al., 2012a; Chung et al., 2012b; Drozdov et al., 2010). In general, only modest differences were observed in enriched genes in Pw1 cells from pregnant and non-pregnant hearts, mainly attributed to the higher level of activation of Pw1 heart cells during pregnancy.
Figure 22. Microarray gene expression analysis of Pw1 cells isolated from pregnant and un-pregnant hearts

(A) Venn diagram of differentially expressed genes from Pw1<sup>eGFP</sup> cells isolated from non-pregnant and 7.5 days pregnant hearts. Analysis reveals 358 commonly expressed genes, 109 genes specific for un-pregnant and 231 genes specific for pregnant Peg3<sup>eGFP</sup> cells. (B) KEGG and Panther pathway analysis of enriched genes in Peg3<sup>eGFP</sup> cells from pregnant hearts. Enriched genes are involved in protein processing in endoplasmic reticulum, regulation of inflammation and signalling pathways implicated in EMT and physiological heart hypertrophy (TGFβ, MAPK, PI3 kinase and integrin signalling pathways).
For studying the role of Pw1 heart cells *in vivo* I took advantage of the Pw1\(^{nLacZ}\) reporter line to specifically inhibit β-gal positive cells using pharmacological approach. We used “Daun02 inactivation method” previously described for specific inactivation of cocaine induced psychomotor activity of nucleus accumbens neurons in rats (Koya et al., 2009). Daun02 is a substrate for β-galactosidase activity in which Daun02 is cleaved into daunorubicin, reducing cellular activity (Farquhar et al., 2002). As Daun02 has poor distribution beyond the vascular space I administrated the drug dissolved in 1% of sodium alginate and injected directly into the pericardial space. I treated four groups of animals: wild type and Pw1\(^{nLacZ}\), non-pregnant and 7.5 days pregnant mice. Seven days post-treatment mice were sacrificed and tissue was collected for histological analysis. Comparing the body weight and heart weight of treated animals after normalization to tibia length I noticed reduction in heart growth during pregnancy in Pw1\(^{nLacZ}\) animals treated with Daun02 (Figure 23b). Body weight increase during the pregnancy between all groups was normal (Figure 23a).

**Figure 23.** Comparison of body and heart growth rates during pregnancy after inactivation of cardiac Pw1 cells with Daun02
(A) Body weight to tibia length ratio of non-pregnant and 14.5 days pregnant animals shows normal growth rate of the Daun02 and PBS treated animals. (B) Heart weight to tibia length ratio in pregnant animals treated with Daun02 is reduced in comparison with the animals treated with PBS. Values are presented as the mean ± SEM, n=4

Histological analysis after H&E staining revealed cardiomyocyte atrophy of the compact myocardium near epicardial surface (Figure 24). Cardiomyocytes of non-pregnant Pw1<sup>nLacZ</sup> animals treated with Daun02 were smaller and lacking normal organization in comparison to the wild type animals (Figure 23a left panel). During pregnancy cardiomyocytes show hypertrophic growth in wild type and Pw1<sup>nLacZ</sup> animals (Figure 24a right panel). In pregnant Pw1<sup>nLacZ</sup> animals treated with Daun02 hypertrophic growth of cardiomyocytes is unequal and disorganised with small pockets of fibrosis at the surface of the heart (Figure 24b).

**Pw1 regulates signalling through regulation of growth factor receptors**

Recently, Thiaville and colleagues (2013) showed by chromatin immunoprecipitation (IP) that PW1 directly binds to DNA and regulates transcription of growth factor signalling adaptor proteins Grb10 and Dusp1. Furthermore, these data were confirmed in Pw1<sup>DMR</sup> mutant mice with lower expression of PW1 protein. To investigate if PW1 protein can directly up-regulate expression of Grb10 and Dusp1 I generated a construct for transient and stable over-expression, where PW1 protein is labelled with HA-tag at its N-terminus (Figure 10). First, I checked if the construct gives functional expression of PW1 protein in HEK 293 cells by immunohistochemistry and Western blotting (Figure 25). In transfected HEK 293 cells PW1 protein was detected in nucleus (Figure 25a), which is in accordance with its predicted transcription factor role (Relaix et al, 1996). Furthermore, with Western blotting using anti-HA-tag and anti-PW1 antibody I showed that PW1 protein is expressed at its full length (Figure 25b).
Figure 24. Daun02 treatment of Pw1
\textsuperscript{nLacZ}
 mice causes local atrophy and fibrosis in non-pregnant and pregnant animals.

\(\beta\)-galactosidase can catalyze the conversion of the prodrug Daun02 into daunorubicin, which reduces cellular activity. Administration of Daun02 into pericardial sac of wild type and Pw1
\textsuperscript{nLacZ}
 causes local atrophy and cardiomyocyte disorganization in transgenic animals (A) and local fibrosis in pregnant animals (B).
Figure 25. HEK 293 cell line transfected with CMV:HA-Pw1 plasmid show strong PW1 protein expression with nuclear localization

Transiently transfected HEK 293 cells with CMV:HA-Pw1 showed high expression of PW1 protein with immunohistochemistry and Western blot analysis. (A) Using the advantage of HA tag PW1 protein was detected in nucleus of transfected (T) cells. (B) Western analysis of PW1 protein expression. 20µg of protein were loaded for each sample. CMV:HA-Pw1 transfected cells (T) show high levels of PW1 detected with anti-HA tag and anti-PW1 antibodies in comparison with untransfected controls (UT).

To investigate if over-expression of PW1 protein can directly up-regulate the expression of Grb10 and Dusp1 over time I generated a stable NIH 3T3 cell line, which expresses high levels of PW1 and RFP under the pTRE3G promoter after doxycycline treatment. As cardiac Pw1 cells express high levels of Igf1 (Figure 18) and as IGF1 signalling is necessary for normal heart maintenance and growth I also looked for expression of Igf1 and Igf1r in PW1 over-expressing cells (Figure 26). NIH 3T3 cells were grown to 80% confluence before they were treated with doxycycline for 24 and 48h. Positive and negative cells for RFP were sorted by FACS (Figure 26a) and total RNA was isolated as described in Materials and Methods. Relative expression levels of Pw1, Grb10, Dusp1, Igf1 and Igf1r were analyzed by qRT-PCR using
TaqMan probes (Figure 26b). Results showed higher levels of Pw1 expression in RFP positive cells (approximately 60,000 fold) than in control after 24h on doxycycline. After 48h expression levels of Pw1 were reduced but were still significantly higher (approximately 20,000 fold) than in the control cells. Higher levels of expression of adaptor proteins Grb10 and Dusp1 were accompanied by up-regulation of Pw1. Unexpectedly a slight up-regulation of Igf1r (approximately 6 fold) was noted in Pw1 expressing cells; as well as higher levels of Igf1 (up to 20 fold). Higher levels of Igf1 were in accordance with up-regulation of adaptor proteins Dusp1 and Grb10 which slow down IGF1 signalling and increase IGF1 secretion by preventing negative feedback loop through IGF1R inactivation.
Figure 26. Relative expression of Pw1 and its target proteins Grb10 and Dusp1 in inducible Pw1 over-expressing NIH 3T3 cells.

Inducible Pw1 system was generated by electroporating pTRE3G: Pw1 plasmid into TetOn NIH 3T3 cell line. (A) After doxycycline treatment cells that integrate pTRE3G: Pw1 plasmid express RFP and PW1 and based on RFP expression can be sorted by FACS. (B) Expression of Pw1, Grb10, Dusp1, Igf1R and Igf1 was measured by qRT-PCR in sorted NIH 3T3 RFP(+) and as a control RFP(-) cells after 24 and 48h of doxycycline treatment.
4. Discussion

The imprinted genes play a crucial role in development of placental mammals through regulation of nutrient transfer from the mother to the offspring. Paternally expressed genes generally enhance foetal growth whereas maternally expressed genes suppress foetal growth and the balance between the two is responsible for the setting the normal metabolic rate of development. The majority of imprinted genes are highly expressed during the development and early postnatal life, but then their expression becomes restricted to tissue specific cells (Lui et al., 2008). As a part of the metabolic network recently it was shown that in adulthood expression of many imprinted genes is restricted to the stem cell compartment. In the hematopoietic system imprinted genes are expressed in long term hematopoietic stem cells, in skeletal muscle they are expressed in satellite cells and their progenitors, and in skin in epidermal stem cells (Berg et al., 2011). Furthermore their expression is rapidly altered after perturbations such as 5-fluorouracil stimulation in hematopoietic system or cryo-injury in skeletal muscle (Berg et al., 2011; Mitchell et al., 2010) that stimulate the stem cell compartment.

As a part of so-called imprinted gene network Pw1 is expressed in the majority of adult stem cell compartments in bone marrow, brain, skeletal muscle, skin and intestines (Besson et al., 2011). To investigate if Pw1 also mark stem cell compartment of adult heart where they appear in significant numbers, I investigated the gene expression profile, morphology and embryonic origin of cardiac Pw1 cells. Although cardiac Pw1 cells express some stem cell receptors such as Cd29, Cd34, Sca1 and Pdgfra from their morphology and behaviour during pregnancy it seems “stemness” is not their primary role. These data are in accordance with recently published studies showing that regenerated cardiac muscle in lower vertebrates and early postnatal mammals comes from pre-existing cardiomyocytes rather than from cardiac stem cells (Poss et al., 2002; Porrello et al., 2011).

Pw1 cells appear in the heart between developmental stages E9.5 and E10.5 what is the time when epicardium is formed and heart is invaded with EPDCs (epicardium derived cells). Adult cardiac Pw1 cells express epicardial
markers (Upk3b, Wt1, Raldh2, Tcf21 and Tbx18) and EMT (Snail, Slug, Twist1) shown by qPCR. Based on timing during development and on these gene expression profiles I surmise a proepicardial/epicardial origin of cardiac Pw1 cells. Although it was shown that during development epicardial cells can give rise to the majority of cardiac cell types, in adults the role of epicardium as a source of cardiac progenitors still remains very controversial. Using developmental genes such as Tcf21, Wt1 or Pdgfra as markers of activated adult epicardial cells it was shown that EPDCs in vitro have trans-germ layer potency (Smart et al., 2007; Chong et al., 2011). Because of the lack of exclusive adult epicardial markers this claim of multi-potency in vivo is at least questionable. In a recent study using more mature marker for epicardial cells Upk3b, Huang and colleagues (2012) showed the importance of epicardium in modulating immune processes in injured heart through its paracrine role. In similar manner through secretion of growth and survival factors (FGF2, IGF1, VEGFA) epicardial cells reduced scaring in post-injured heart (Zhou et al., 2011). These new data suggest an important role of the epicardium in modulating cardiac environment by secreting various growth factors rather than acting as a source of progenitors in injured muscle.

Based on our data and the data of others mentioned before I hypothesise that the primary role of epicardium is not to provide a source of progenitors in adult hearts. What is clear from my microarray data on cardiac Pw1 cells is that the epicardium can go through EMT process throughout adulthood and this developmental program could be responsible for stem cell phenotype of adult EPDCs. Recently was shown that in breast epithelia the tumour suppressor p53 has a role in regulating both EMT and EMT-related stem cell properties through activation of miR-200c (Chang et al., 2011). Not only is EMT connected to stemness in various cancer models (Mani et al., 2008; Chiou et al., 2010; Li and Zhou, 2011; Tellez et al., 2011), but also in generating proliferative islet precursor cells in pancreas (Gershengorn et al., 2004). Furthermore knock out of Wt1, a reporter of activated epicardium, in embryonic stem cells caused reduction of EMT, impairment in cell migration and differentiation of embryoid bodies into hematopoietic, endothelial and cardiac lineages (Martinez-Estrada et al., 2010). This study and others reveals a potential therapeutic implication of EMT not only for cancer
treatments but also for regeneration. Using naturally occurring or induced EMT of the adult epicardium and applying known growth factors for differentiation of EPDCs from *in vitro* studies could have great potential in healing injured myocardium in the near future.

An important function of foetal epicardium is to regulate myocardial growth and angiogenesis (Pennisi and Mikawa, 2009; Brade et al., 2011; Chen et al 2002). Studies using FGFR1 and FGFR2 knock out mice have shown the importance of epicardial derived FGF signals for direct and indirect myocardial proliferation and coronary growth during midgestation heart development (Lavine et al., 2005; Lavine et al., 2006). Zhou and colleagues (2011) using Wt1 to delineate activated epicardium described proangiogenic role of EPDCs in injured heart in adult animals. Using blocking antibodies they showed that Fgf2 and Vegfa accounted for approximately half the growth-promoting activity in post MI EPDCs. Furthermore EPDC secreting factors from *in vitro* conditioned media reduced infarct size and improved heart function.

The GO Ontology and pathway analysis of Pw1 signature genes revealed a potential role of Pw1 cells in cardiac remodelling. Genes involved in angiogenesis, ECM synthesis and organization and cell adhesion are more consistent with a cell type responsible for maintaining the structure of the heart than with a stem cell giving the rise other cell types. Furthermore Pw1 cells express numerous growth factors such as Vegfa, Jag1, thymosin β4, angiopontins and growth factors from insulin and TGFβ family important for vasculature remodelling and cardiac growth during physiological hypertrophy. Close connections of Pw1 cells with capillaries revealed by immunohistochemistry and the fact that Pw1 cells are intimately associated with endothelial cells in our *in vivo* model of angiogenesis, stressing the importance of these fibroblast like cells in maintaining and remodelling vasculature during cardiac homeostasis.

Together with published data on the paracrine role of epicardium during development and injury, I propose that epicardium-derived Pw1 cells play an important role in heart growth and homeostasis as scaffolding cells for vasculature (Figure 25). Heart hypertrophy, caused by pregnancy or exercise, is accompanied with vessel remodelling and angiogenesis. Muscle
hypertrophy causes local hypoxia and mechanical stimuli for activation of epicardium. Activated epicardial cells, Pw1 cells, migrate in to the cardiac muscle providing the paracine signals, together with structural support to promote vasculogenesis.

Figure 27. Model of the scaffolding function of Pw1 cells
During postnatal heart growth cardiomyocyte hypertrophy is accompanied with vessel remodelling and angiogenesis. Local hypoxia and mechanical stress activates epicardial cells to undergo EMT and start expressing Pw1. Activated Pw1 cells migrate into the muscle where through secretion of growth factors and scaffolding support provide signalling queues for angiogenesis.

Lower vertebrates can completely regenerate injured heart tissue without scarring (Tsai et al., 2007). One of the abilities of lower vertebrates such as zebrafish and amphibians is that they can grow throughout life, which is accompanied with equivalent levels of heart growth (Jordan, 1905). Wills and co-workers (2008) noticed that lowering fish density in the aquarium triggered rapid animal growth and robust cardiomyocyte proliferation throughout the adult ventricle, greater than that observed during slow animal growth or size maintenance. This rapid postnatal cardiac growth is accompanied with activation of developmental epicardial genes including Raldh2 and Tbx18. They also showed that intervention in epicardial activation during this process of zebrafish hypertrophy results in cardiac scarring. Understanding the pathways involved in physiological heart growth could be beneficial for future treatment of pathological conditions.

To determine if similar activation of epicardium happens during rapid postnatal heart growth in mammals, I documented Pw1 expression during pregnancy, which is associated with a prolonged cardiac volume that results in cardiac hypertrophy with no induction of the foetal gene program (Eghbali et al., 2005). Rapid hearth growth during first week of pregnancy is accompanied with higher numbers of epicardial and interstitial Pw1 cells. Looking into specific gene signature of Pw1<sup>eGFP</sup> cells during pregnancy I saw up-regulation in MAPK, TGFβ, Wnt and PI3 kinase signalling pathways, which have been previously described as pathways important for cardiomyocyte growth during physiological hypertrophy (Chung et al., 2012a; Drozdov et al., 2010; Chung et al., 2012b). Furthermore using the Daun02 method for inactivation of Pw1 cells I documented a reduction in heart hypertrophy and scarring during pregnancy, in a similar way to the scarring produced by blocking the FGF signalling during rapid heart growth in zebrafish (Wills et al.,
2008). Daun02 treatment not only caused atrophy in pregnant mice, but also in non-pregnant controls. This is in accordance with the fact that PW1 cells express numerous growth factors and structural materials for normal cardiac homeostasis.

Physiological cardiac hypertrophy caused by post-natal growth, pregnancy or exercise is primarily mediated by IGF1 signalling. Not only is IGF1 important in the context of rapid heart growth, but it is also an important signalling molecule for maintaining cardiac homeostasis. According to microarray expression profile and qRT-PCR Igf1 mRNA is highly expressed (more than 100 fold) in Pw1 cells in comparison to total RNA from the heart. To investigate the potential role of PW1 in IGF1 signalling I investigated transcript levels of Igf1 and Igf1r in cell lines with stable PW1 over-expression. Also I monitored expression of two direct targets of PW1, Grb10 and Dusp1, which were described as regulators of IN/IGF1 signalling.

GRB10 is adaptor protein that interacts with numerous receptor tyrosine kinases like IR/IGF1R (Durfresne and Smith, 2005; Langlais et al., 2004), EGF receptor (Ooi et al., 1995; Rose et al., 1998), PDGF receptor (Frantz et al., 1997; Wang et al., 1999), Ret (Pandey et al., 1995) and the ephrin receptor ELK (Stein et al 1996). The action of GRB10 is best described as negative regulator of insulin/IGF1 mediated signalling through receptor internalization (Stein et al., 2001; Monami et al., 2008). Another PW1 target involved in down regulating IN/IGF1 signalling is DUSP1, a phosphatase that specifically inactivates MAPK pathway (Cellier et al., 2003; Manetsch et al., 2012).

These data show that cells expressing PW1 at high levels also express higher levels of Grb10 and Dusp1. Over time, cells expressing PW1 also express higher levels of IGF1. IGF1 signalling is subjected to negative regulation through feedback mechanisms and higher levels of Grb10 and Dusp1 would prevent this negative feedback loop allowing higher expression of IGF1 over time (Figure 28). We hypothesize that PW1 down-regulates growth factor signalling through direct targets Grb10 and Dusp1 preventing negative feedback and higher secretion of growth factors including IGF1 in epicardial activated PW1 cells. Similarly to PW1 knock-down mice, transgenic GRB10 over-expressing mice show postnatal growth retardation (Shiura et al.,
confirming the role of PW1 and GRB10 in setting the metabolic rate of growth. Furthermore, mice with a disruption of the Grb10 gene had improved whole-body glucose tolerance and insulin sensitivity, as well increased muscle mass and reduced adiposity (Smith et al., 2007; Charalambous et al., 2003), what is in accordance with increased growth factor signalling. Another mechanism through which PW1 could directly inhibit IGF1 signalling is through direct protein interaction with decorin (Buraschi et al., 2013). Decorin binds to surface of several growth factor receptors and negatively regulates their activity and signalling via robust internalization and eventual degradation (Goldoni and Izzo, 2008; Buraschi et al., 2010).

The role of PW1 in regulating growth factor signalling would explain its broad expression in various cell types in adulthood. In heart, liver and pancreas PW1 is expressed in the growth factor producing cells preventing a negative feedback loop, whereas in skeletal muscle and hematopoietic system it is expressed in subpopulation of stem cells preventing their activation and keeping them quiescent in normal homeostatic conditions.

![Schematic representation of regulation IGF-1 signalling by PW1](image)

**Figure 28. Schematic representation of regulation IGF-1 signalling by PW1**

During IGF1 signalling IGF1 binds to IGF1R causing phosphorylation of the receptor and activation of MAPK and mTOR signalling cascade, which activates transcription of target genes. Overstimulation of MAPK and mTOR pathway also inhibits further transcription and secretion of IGF-1. PW1 direct targets GRB10 and DUSP1 inhibit
IGF1 signalling pathway on several levels and preventing negative feedback loop for IGF1 secretion. GRB10 interacts with IGF1R preventing its activation and DUSP1 is phosphatase that inhibits MAPK pathway.
5. Conclusion and Future Directions

In this research using the Pw1 reporter mouse lines, I have described a subpopulation of epicardium-derived mesenchymal cells involved in maintaining the integrity of adult mouse heart through secretion of various growth factors and components of extracellular matrix. Cardiac Pw1 cells express high levels of Igf1, Vegfa, Jag1, angiopoietins, growth factors from TGFβ family and thymosin β4 necessary for cardiomyocyte growth and angiogenesis. I have also shown that Pw1 cells are highly pro-angiogenic in vitro and can induce tube formation when they are co-cultured with an endothelial cell line. Using pregnancy as a model of cardiac growth I have shown that Pw1 cells play an essential role in tissue remodelling during physiological heart hypertrophy. Inhibition of Pw1 cells with Daun02 resulted in local cardiomyocyte atrophy and fibrosis. All these results suggest an important role of Pw1 cells in heart homeostasis and potentially, in formation of various pathological conditions. Previously published data showed the importance of epicardium-derived cells in regulating inflammation and scarring after heart injury (Huang et al., 2012). Based on this previous research and based on my microarray data, where Pw1 cells are enriched in different cytokines, I propose further investigation of interaction between Pw1 cells and immune cells in different models of heart injury.

Immunohistochemistry and cytometric analysis revealed that myocardial Pw1 cells express the cardiac stem cell membrane receptors Sca1, Pdgfra, Cd34 and Cd29, making them a potentially interesting component of regenerative medicine for further investigation. Based on ultra-structural and functional assays it seems “stemness” is not the primary role of Pw1 cells. No less, the differentiation potential of Pw1 cells in in vitro and in vivo conditions should be addressed. Albeit it was shown that newly formed cardiomyocyte in regenerative myocardium do not come from stem cells, but from already existing cardiomyocytes (Poss et al., 2002; Porrello et al., 2011), using naturally occurring or induced EMT of epicardium in generating cardiac progenitors could have great potential in healing injured myocardium.
Although the literature has described different cell types expressing Pw1 in various tissues (Mitchell et al., 2010; Besson et al., 2011; Berg et al., 2011) the role of PW1 protein still remains unclear. PW1 is expressed in majority of quiescent stem cells together with other members of imprinted gene network, setting up the metabolic rate of individual cells through regulation of growth factor signalling. How this regulation works and where in this protein hierarchy PW1 is, still needs further investigation. For these reasons, I propose chromatin IP experiments on Pw1 heart cells to detect PW1 direct transcriptional targets. According to my microarray expression profile and qRT-PCR Igf1 mRNA is highly expressed in Pw1 cells, which makes Pw1 cells an interesting subject for IGF1 signalling studies. Physiological cardiac hypertrophy caused by post-natal growth, pregnancy or exercise is primarily mediated by IGF1 signalling (Chung et al., 2012a; Chung et al., 2012b; Drozdov et al., 2010). Investigating the role of PW1 in cardiac IGF1 signalling, could have a great potential for understanding diseases like post- and peri-partum cardiomyopathies which lead to complete organ failure and are induced by defects in cardiac growth during pregnancy. Generating inducible Pw1 knock out line and investigating deletion of IGF1 specifically in cardiac Pw1 cells would be of great interest for understanding the role of Pw1 cells in regulating cardiac homeostasis.
6. Literature


Cardiac progenitor cells and biotinylated insulin-like growth factor-1 nanofibers improve endogenous and exogenous myocardial regeneration after infarction. Circulation 120 (10): 876-87.


