Characterization of mesenchymal stem/progenitor cells and their progeny from non-fetal tissue sources

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Summary

Damage to a tissue, in suboptimal settings, such as post-fetal wound healing, results in a reparative process characterized by varying degrees of inflammation, aberrant blood vessel formation and fibrosis, which eventually leads to a decay in organ function resulting in clinical symptoms. Synthesis of collagen type I by fibroblast, the dominant component of the extracellular matrix, is crucial in tissue repair and the pathogenesis of fibrotic disease. Thus, modulation of fibroblast behavior may be a target to reduce fibrosis and optimize organ repair processes. Pericytes, by definition, are mesenchymal cells localized juxtapositioned to the abluminal side of endothelial layer in capillaries, venules and small arterioles, and encased in the microvascular basement membrane. They are believed to be important regulators of blood vessel function and angiogenesis. Previous studies suggest that pericytes may be the progenitor of fibroblast. Pericytes have also been further hypothesized as mesenchymal stem cells. The main objective of the project was to identify the progenitor of collagen type I producing fibroblasts with special reference to pericytes.

Pericytes were isolated from human placenta based on their in vivo definition. Immunofluorescent staining and immunohistochemical staining were used to further characterize the marker expression profile. Flow cytometry was employed to analyze marker expressions of pericytes in different serum concentrations as well as the influence of platelet derived growth factor-BB and transforming growth factor-β stimulation on the differentiation process. Western blot analysis was employed here to examine the production of procollagen type I in cells in different stages of the differentiation.

Pericytes in non-serum condition showed a tendency to maintain the pericyte phenotype. No apparent influence of platelet derived growth factor-BB and transforming growth factor-β on the differentiation process was found. Procollagen type I production of cells in different stages supported the hypothesis that placenta pericytes derived from MVFs differentiated into collagen type I producing fibroblasts.
## Abbreviations

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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>MVF</td>
<td>Microvascular fragment</td>
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<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<td>TGF</td>
<td>Transforming growth factor</td>
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<td>MSC</td>
<td>Mesenchymal stem cell</td>
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<td>α-SMA</td>
<td>α-Smooth muscle actin</td>
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<td>FCS</td>
<td>Fetal calf serum</td>
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<td>FSP</td>
<td>Fibroblast surface protein</td>
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<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
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Introduction

Tissue repair and fibrosis
Damage to tissue starts with inflammation, followed by a tissue repair process that consists of a series of events including proliferation and migration of cells, formation of new blood vessels, production of extracellular matrix (ECM) and tissue remodeling. In optimal settings, such as fetal wound healing, these result in reinstatement of tissue architecture with retained function of the organ and minimal fibrosis (Buchanan et al., 2009). In post-fetal wound healing, which is often suboptimal, these result in a reparative process characterized by varying degrees of inflammation, aberrant blood vessel formation and fibrosis, which eventually leads to a decay in organ function resulting in clinical symptoms (Darby and Hewitson, 2007). Fibroblasts are the main source of collagen type I, the dominant component of the extracellular matrix, which is of central importance in tissue repair, and in the pathogenesis of fibrotic disease. Thus, modulation of fibroblast behavior may be a target to reduce fibrosis and optimize organ repair processes.

The mechanism of scarless wound healing remains largely unknown. Studies suggest a number of differences between scarless and scarring wound healing. A higher ratio of collagen type III to type I was found in fetal skin (Merkel et al., 1988). Scarring wounds have more active myofibroblasts, which relates to the degree of scarring (Estes et al., 1994). Hyaluronan is the main component of fetal ECM. High level of hyaluronan continue in fetal wound healing, while it is just initially deposited in adult wound healing and then is quickly replaced by collagen (Clark, 1989; Krummel et al., 1987). The key observation is that fetal/scarless wounds heal rapidly with a paucity of inflammatory cells. Moreover, introduction of inflammation into fetal wounds lead to increases in wound macrophages, neutrophils, collagen deposition and scarring (Frantz et al., 1993). The later suggests inflammation including cellular inflammatory mediators, cytokines, etc. play an important role in scar formation. Compared to fetal platelets, adult platelets produce more platelet-derived growth factor (PDGF), and transforming growth factor (TGF)-β1 and β2 (Olutoye et al., 1996). Fetal platelets also show reduced ability of aggregation (Olutoye et al., 1997a; Olutoye et al., 1997b). Neutrophils are recruited to the site of injury by TGF-β1 and PDGF. They release cytokines and chemotactants for fibroblast and macrophages. Fewer macrophages were found in fetal wounding (Singer and Clark, 1999). Fibroblasts play a crucial role in synthesis and remodeling of ECM. In vitro, fetal fibroblast synthesize more collagen type III and IV than the adult counterparts (Krummel et al., 1988; Lorenz and Adzick, 1993). In adult wounds, collagen synthesis is delayed when fibroblast proliferate, while fetal fibroblasts proliferate and synthesize collagen simultaneously (Clark 1996). Not only the concentration but also the ratio of certain growth factors play an important role in the wound healing process. TGF-β is chemotactic for fibroblast and stimulates fibroblast production of collagen type I. TGF-β1 and β2 isoforms expression increase in adult wounds and exogenous administration of these isoforms to adult wounds increases collagen deposition and inflammatory cell accumulation. The ratio of TGF-β3 to TGF-β1 has been suggested to determine whether tissues regenerate or form scars. In scarless fetal wound healing TGF-β3
expression is increased and TGF-β1 expression is unchanged. In contrast, in scarring tissue, TGF-β1 expression is increased and TGF-β3 decreased (Beanes et al., 2001; Hsu et al., 2001). VEGF expression increased faster in scarless wound healing model than that seen in adult scarring (Colwell et al., 2005), which may contribute to the relatively faster healing process in scarless wounds. PDGF-BB, one of the isoforms of dimeric PDGF, in vitro, is a mitogen and chemoattractant for mesenchymal cells including fibroblast (Whitby and Ferguson, 1991). PDGF-BB, in vivo, increases the number of macrophages and fibroblast as well as stimulating fibroblast migration into wounds, and appears to enhance the inflammatory phase thereby indirectly triggering procollagen type I synthesis (Pierce et al., 1991a; Pierce et al., 1991c).

**Pericyte**

Pericytes are defined as mesenchymal cells localized juxtapositioned to the abluminal side of endothelial layer in capillaries, venules and small arterioles. Pericytes are encased in the microvasular basement membrane (Rhodin, 1968). Pericytes are in direct cell-cell contact and partially cover the endothelial cells. The pericyte to endothelial cell ratio shows substantial differences depending on tissue type, vessel type and state of activation of the tissue (Allt and Lawrenson, 2001). Pericytes also show differences in morphology based on tissue location and type of vessel. Normally, they show an elongated, branched morphology with prominent nuclei and limited perinuclear cytoplasm.

It is difficult to identify pericytes only based on cell-type specific pericytes markers, due to the diverse characteristics, locations and functions. In vivo, they are usually identified by the morphology, spatial relation to endothelium, in conjunction with the expression of a combination of several markers. This is more complicated in vitro, due to the loss of spatial and morphological cues in culture. There are several markers commonly used to identify pericytes: PDGFR-β, a cell surface receptor used as a pericytes marker in activated tissue (Sundberg et al., 1993); α-smooth muscle actin (α-SMA), used as a marker for mature pericytes, which is a cytoskeletal protein also expressed by smooth muscle lineages and myofibroblast (Skalli et al., 1989); Neuro-glial 2 (NG2), corresponding to high-molecular weight melanoma-associated antigen in humans, which is a chondroitin sulphate proteoglycan expressed in activated arteriolar, capillary and venules pericytes (Ozerdem et al., 2001); and desmin, a muscle-specific class III intermediate filament (Nehls et al., 1992). None of these are general pericyte markers, and their expression profiles vary in different tissues and the activation state of blood vessels.

In the vasculature, pericytes are in direct cell-cell contact with the endothelial layer and are believed to be important regulators of blood vessel function and angiogenesis. Pericytes can cause vasoconstriction and vasodilation within capillary beds and thereby regulate capillary blood flow (Rucker et al., 2000). They are also involved in vascular pruning, vessel architecture regulation and structural and functional vessel stabilization (Hellstrom et al., 2001). Angiogenesis is the formation of new blood vessels from existing ones. Genetic ablation of either PDGF-BB or PDGFR-β in mice embryos results in perinatal lethality due to severe hemorrhage (Leveen et al., 1994). These mouse embryos lack microvascular pericytes, suggesting a crucial role of pericytes in capillary wall stability (Lindahl et al., 1997). During
angiogenic sprouting, PDGF-BB is expressed by endothelial cells and PDGFR-β is expressed by pericytes (Sundberg et al., 1993), indicating paracrine signal interactions between these two cell types (Hellstrom et al., 1999). Through direct contact between these two cell types, pericytes can regulate endothelial proliferation and differentiation (Hellstrom et al., 2001; Hirschki et al., 1999).

Pericytes show tissue-specific functions in different organs and therefore have been given additional names in these organs. Blood vessels in neural tissues including the brain and the retina exhibit the highest density of pericytes in the body. These pericytes play an essential role in the structural integrity of these vessels and are important in maintaining the blood-brain barrier (Hayashi et al., 2004). Pericytes in the brain also perform macrophage-like activities, which raised the hypothesis that pericytes are macrophage precursor cells in the brain (Thomas, 1999). Liver pericytes are usually called hepatic stellate cells (HSCs) or Itoh cells. In liver the endothelial lining in vessels is discontinuous, and HSCs create cell-cell contact with endothelial cells through paucities in the basement membrane. HSCs can regulate ECM remodeling via producing ECM components and matrix metalloproteinases (Friedman, 2000). They are the main producer of collagen type I and important in liver fibrosis. HSCs store more than 80% of total vitamin A in the body and participate in vitamin A metabolism (Higashi et al., 2005). Moreover, these cells are involved in inflammatory cell recruitment during hepatic tissue repair on the one hand and liver diseases resulting in fibrosis on the other (Knittel et al., 1999). Mesangial cells, pericytes of glomerular capillaries in kidney, create a significantly increased capillary surface area for blood ultrafiltration (Betsholtz et al., 2004).

**Pericyte-fibroblast transition**

It has been hypothesized that pericytes function as progenitors for collagen type I producing fibroblasts (Ivarsson et al., 1996; Rodriguez et al., 2009; Sundberg et al., 1997; Sundberg et al., 1996; Sundberg et al., 2002; Sundberg et al., 1993; Sundberg and Rubin, 1996). Fibroblasts are normally in a quiescent state, and able to differentiate to myofibroblasts in tissue repair. Other possibilities regarding the cellular origin of interstitial fibroblasts include resident fibroblast populations, circulating precursors, epithelial cells and endothelial cells (Darby and Hewitson, 2007). Evidence supporting a pericyte origin for fibroblasts include (1) phenotypical markers that are common for pericytes and myofibroblasts in for instance dermal fibrosis (Sundberg, Ivarsson et al. 1996; Sundberg, Kowanetz et al. 2002), (2) fate tracing experiments which reveals that pericytes are myofibroblast progenitors in fibrotic kidney (Humphreys et al., 2010), (3) observations that pericytes from human placenta, an organ with little collagen type I synthesis, can differentiate into collagen type I producing cells in vitro (Ivarsson et al., 1996), (4) the observation that fibroblasts bear PDGFR-β receptors in vitro and respond to PDGF-BB stimulation by increased proliferation and migration (Pierce et al., 1991b), and (5) the fact that PDGFR-β is expressed on pericytes in vivo and in vitro (Sundberg et al., 1996; Sundberg et al., 1993).

**Stem cells**

Stem cells are usually described as cells which have the potential to differentiate into a range
of specialized cell types (multi-potent). It is a challenge to characterize stem cells \textit{in vivo} due to a lack of markers and the difficulties related to cell fate tracing, as well as \textit{in vitro} due to a loss of stem cell characteristics secondary to the culturing environment. Certain criteria must be fulfilled as a cell to be termed a “stem cell” which include: lack of expression of differentiation markers; capability of proliferation; ability to self-maintain their own cell population; ability to produce a large number of differentiated, functional progeny cells; and being able to regenerate tissues after injury. “Transit cells”, a differentiation state between stem cells and mature cells, are characterized by: the onset of expression of differentiation markers, which however is not mandatory; a marked ability to proliferate and expand the cell population; and the inability to self-maintain the cell population. “Maturing cells”, cells in the final steps of differentiation, are defined as cells that express numerous differentiation marker, cannot or only to a limited extent can proliferate, lack the capability of self-maintenance, acquire specialized cellular functions which are often tissue specific; and lack the capability to produce any progeny after tissue injury, hence lack the ability to regenerate tissue after injury (Potten 1997).

Mesenchymal stem cells (MSCs) are described as plastic-adherent pluripotent cells with the ability to generate different cell-types constituting the mesenchymal cell lineages including osteoblasts, chondrocytes, fibroblasts, adipocytes and others. They can be isolated from many types of adult tissue, including bone marrow, umbilical cord, adipose tissue and placenta. Stro-1 is the best-known MSC marker so far. cd146 has also been suggested as a marker for MSC derived from multiple organs (Kolf et al., 2007; Liu et al., 2009). Both \textit{in vitro} and \textit{in vivo} studies showed that pericytes can differentiate into smooth muscle cells, collagen type I producing fibroblast, myofibroblast, as well as osteogenic, chondrogenic, adipogenic, myogenic cell lineages (Crisan et al., 2008; Diaz-Flores et al., 1991). Therefore, pericytes are suggested to act as pluripotent mesenchymal stem cells. The study of pericytes has been hampered by the fact that pericytes are not phenotypically stable in culture. This is the main focus of the current investigation (Ivarsson et al., 1996; Sundberg et al., 2002).
**Aim**

The main objective of the project was to identify the progenitor of collagen type I producing fibroblasts *in vitro*. The specific aims are to 1) isolate microvascular pericytes from tissue, 2) propagate pericytes *in vitro*, 3) stabilize pericyte phenotype in culture, 4) characterize the differentiation pathway from pericytes to collagen type I producing fibroblast *in vitro*, and 5) investigate if PDGF-BB and TGF-β influence this process.
Results

Isolation of microvascular fragments from placenta
To isolate pericytes according to their *in vivo* definition, human placenta was chosen because it is an organ rich in blood vessels and with little native collagen synthesis which means it has few fibroblasts by definition. The later results in a weaker background for studying the differentiation process of pericytes to collagen type I-producing fibroblasts. Microvascular fragments (MVF s) were isolated from human placenta by mincing the tissue, digesting the tissue with collagenase, filtering away debris and larger vessel fragments, and washing away red blood cells. MVFs mainly contain pericytes and endothelial cells (Challier et al., 1995).

The MVFs attached to the collagen coated flasks 2 or 3 days post-seeding. At this timepoint pericytes started to emerge from MVFs after attachment. Cells continued to emerge and expand from MVFs and reached confluency in 25 cm² culture flask in about 2 weeks (Fig. 1).

Immunolabeling of pericytes derived from placental and foreskin MVFs
To characterize the marker expression profile placental pericytes and if this marker profile expression was specific for placental pericytes, placenta pericytes and pericytes isolated from foreskin using a similar methodological approach were used. Immunolabeling for light microscopy as well as immunofluorescence was performed on pericytes derived from foreskin and placenta. The marker expression profile showed similar patterns between pericytes from these two sources, and as a crude population there are cells expressing marker for different stages of differentiation (Table 1, Fig 2).

Table 1. Immunolabeling of cell-type specific markers of pericytes in primary cultures

<table>
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<tr>
<th></th>
<th>skin pericytes (isolate 1)</th>
<th>skin pericytes (isolate 2)</th>
<th>placenta pericytes (isolate 3)</th>
<th>placenta pericytes (isolate 4)</th>
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<tr>
<td><strong>Endothelium</strong></td>
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<tr>
<td>Cd31</td>
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<td><strong>Pericyte</strong></td>
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<td>Desmin</td>
<td>+++</td>
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<td>α-SMA</td>
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<td>NG2</td>
<td>+++</td>
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Figure 1. a) Newly isolated MVF. b) Cells emerging from MVF. c) Cells emerging and expanding from MVF. ×100 magnification.
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<tr>
<td><strong>PDGFR-β</strong></td>
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<td><strong>Stem/progenitor</strong></td>
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<tr>
<td>stro1</td>
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<td>+</td>
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<td>cd146</td>
<td>+</td>
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<td><strong>Fibroblast</strong></td>
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<td>FSP</td>
<td>+++</td>
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<td>Procollagen</td>
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<td>type 1</td>
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<td>SB5</td>
<td>+++</td>
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<tr>
<td><strong>Epithelium</strong></td>
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<tr>
<td>Cytokeratin</td>
<td>-</td>
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- negative; +, <30% of cells positive; ++, 30%-70% of cells positive; +++; >70% of cells positive
Figure 2. Immunolabeling of antibodies recognizing markers that are cell-type specific and that depict different stages of differentiation of pericytes/fibroblasts and analyzed using light and fluorescent microscopy of cultured cells. ×200 magnification

**Markers expression profile of pericytes cultured in 10% fetal calf serum compared to serum-free medium**

Microvascular fragments from one placenta were cultured in RPMI containing 10% FCS until the cells had reached the second passage. These cells were then cultured separately in RPMI containing 10% FCS and N2B27 containing no serum. Cells were harvested on 3 day and 7 day time points. Flow cytometry was used to analysis the markers expression profile. Two time points were set to see whether there would be temporal differences in expression marker
When comparing marker expression profiles in cells grown under non-serum conditions to cells grown in 10% FCS, the former expressed less procollagen, PDGFR, cd146 and more α-SMA (Fig 3). Differences in marker expression between the two groups were more apparent at the 7 day compared to the 3 day time point.

Figure 3. Marker expression in cells cultured in 10% serum and serum-free conditions since passage 2. a) Marker expression at 3 day time point; and b) Marker expression at 7 day time point.

Markers expression profile of cells derived from microvascular fragments cultured in 10% serum, 1% serum compared to serum-free medium

Usually, MVFs are cultured in RPMI with 10% FCS. To test if culturing conditions had a larger phenotypical influence at earlier stages of cell culture, MVFs from one placenta were
cultured in RPMI with 10% FCS until MVFs had adhered to the collagen coated dishes. Upon adherence of the MVF’s the medium was changed to N2B27 (non serum), RPMI containing 10% FCS, or RPMI containing 1% FCS. The cells survived and proliferated in all three medium. Cells were allowed to grow to passage 2 whereupon cells were collected and flow cytometry was employed to examine the marker expression profile.

Compared with 10% FCS, both 1% FCS and non-serum cultured cells showed higher expression of stro1, cd146, and PDGFR. Cells in non-serum conditions expressed less α-SMA and procollagen compared to 10% FCS cultured cells; 1% FCS cultured cells had higher and similar expression of α-SMA and procollagen comparing to 10% FCS cultured cells (Fig. 4).

![Marker expression in medium with different serum concentration](image)

Figure 4. Markers expression of passage 2 cells from cells derived from MVFs that upon adherence had been cultured in 10% serum, 1% serum and serum free (N2B27) conditions

**PDGF-BB and TGF-β stimulation**

Passage 2 cells having been cultured in RPMI with 10% FCS were cultured separately in three medium conditions: RPMI containing 10% FCS, RPMI containing 1% FCS, and N2B27 (non-serum). For each medium condition, cells were divided into three groups: PDGF-BB stimulation, TGF-β stimulation and negative control without any foreign source of growth factors. Cells were harvested on 7 day, 14 day, and 21 day timepoints. Markers expression profiles were examined via flow cytometry. No distinct marker expression profile was observed between these two growth factors stimulation. However differences between stimulated and unstimulated cells were observed: in medium containing 1% FCS, when compare with unstimulated cells, neither PDGF-BB nor TGF-β had an influence on markers expression profiles in 7 days and 14 days timepoints. Until 21 days, certain differences appeared: unstimulated cells had higher expression of cd146, PDGFR, α-SMA and procollagen (Fig. 5).
Western blot analysis of procollagen expression in cell subpopulations

Parallel experiments showed that three different stages of differentiation from presumed MSC to collagen type I producing fibroblasts via the pericyte could be discerned termed subpopulation 1, 2 and 3 (data not shown). Subpopulation 1 is stro1 positive/cd146 positive cell population. Subpopulation 2 which was stro1 negative/cd146 positive and subpopulation 3 which was stro1 negative/cd146 negative were isolated via cell sorting. Immunoprecipitates of cell-lysates were run on an SDS-PAGE gel, Western Blotted and labeled with an antibody directed against procollagen type I. Lysate from tumor epithelium cells were chosen as a negative control.

All three samples showed a band of the correct molecular weight between 130 KDa and 250 KDa (Fig. 6). The intensity of the signal in subpopulation 2 (lane 3) has much weaker compared to subpopulation 3 (lane 4). Surprisingly, the signal in tumor epithelial cells (lane 1), which are supposed are not supposed to express procollagen type I and was used as a negative control, showed similar signal intensity compared to subpopulation 3 (lane 4).
Discussion

Pericyte identification *in vivo* and *in vitro* is hampered since there is no general pericyte marker, and it is more complicated *in vitro* than *in vivo* because the spatial relationship with endothelium which can help identification *in vivo* can not be observed in cell culture. To study pericytes *in vitro*, the first problem is to find a suitable approach to isolate these cells. We chose to isolate according to their association to the endothelium (Rhodin, 1968). Microvascular fragments were isolated, which allowed for the spatial distribution to the endothelium to be used as a criteria for identifying pericytes. The human placenta is a tissue source rich in blood vessels. It is also an organ with little collagen synthesis, which leads to a relatively weak background for studying the differentiation from pericytes to collagen type I producing fibroblasts. Therefore, placenta is a good source and was chosen for the project.

Pericytes have tissue-specific characteristics and functions that can also effect marker expression. Pericytes derived from foreskin and placenta were further characterized via a panel of cell-type specific as well as differentiation markers. In these two tissue sources, pericytes were both derived from MVFs. Similar marker expression profiles were found (Table 1). This validates the approach by which pericytes can be isolated. This also indicates pericytes in these two tissues share phenotypical characteristics, and that the results from placenta pericytes are not organ specific but can be extended to skin pericytes and possibly even pericytes from other organs.

Since we studied the differentiation process based on examining the shift in marker expression profile over time and in reduced serum and non serum conditions it is critical to identify and standardize the starting cell population. The number of cells that can be obtained from one placenta is limited, and isolations from many placentas certainly introduce biological variations into the system. To dispel biological variation in this phase of the project, comparison of marker expression patterns were performed on cells derived from individual placentas, and for each individual placenta, a group of cells cultured in 10% FCS was always used as a standard control.

Earlier studies suggested pericytes might act as mesenchymal stem cells that have the potential to differentiate to collagen type I producing fibroblast (Diaz-Flores et al., 1991; Ivarsson et al., 1996; Lin et al., 2008; Sundberg et al., 1996; Sundberg et al., 1993). Based on temporal marker expression profiles, the results support that the differentiation process starting from newly emerging cell from MVFs can be generally divided into three stages: mesenchymal stem/progenitor cell stage, expressing stro1 and cd146; pericytes stage, expressing cd146 but not stro1 and fibroblast stage, negative for stro1 and cd146. Markers used in these experiments were: stro1, being considered as the earliest expressed marker; cd146, a marker expressed later than stro1 on in the differentiation process; PDGFβ and α-SMA, two of the common pericytes markers; and the fibroblast surface protein (FSP) and procollagen type I belonging to markers expressed during the later stages of the differentiation process i.e. the fibroblast stage. Thus the results using the above mentioned markers could temporally delineate three different stages of differentiation from MSC to collagen type
I-producing fibroblast via the pericyte.

Animal serum is widely used in cell culture. It has various components difficult to standardize like cell matrix proteins and various types of growth factors. The main problem of serum in stem cell studies is that serum contains components not only for cell survival and proliferation but also for differentiation. Thus, a serum-free culture condition with defined growth factors is preferred. N2B27 is such a medium. Cells cultured in N2B27 medium was able to partially inhibit the differentiation process toward collagen type I-producing fibroblast indicating that serum-free condition may maintain cells in a certain stage or at least show down the differentiation process (Fig. 4). However, further experiments have to be conducted in order to successfully maintain a phenotypically stable culture of cells in the distinct stages of differentiation.

Differentiation from stem cells to specific specialized cell types is always considered as a one-way process. Initial experiments using passage 8 cells did not show differences in marker expression profile in cells cultured in N2B27 compared to cells cultured in 10% FCS. This might be explained by that the cells that had grown in 10% FCS environment for some time might already to a large extent have differentiated into more mature phenotypes. Thus, an inhibitory effect of N2B27 on this differentiation process would not be detected. Thus, the results suggest that serum-free condition may be able to slow down the differentiation process, but not to drive or revert the cells back to an earlier differentiation stage supporting the notion that the process under study is a one-way process.

In order to minimize the influence of serum on the differentiation process the medium for MVFs were changed into 1% FCS, N2B27 (non-serum) at a much earlier timepoint in the primary cell cultures right after emerging cells appeared. In this case, compared to cells in 10% FCS, passage 2 cells cultured under non-serum condition expressed less α-SMA and procollagen type I and more stromal cell marker 146 and PDGF receptor β, which suggest that cells in non-serum medium were in an earlier differentiation stage compared to cells in 10% FCS. Cells cultured in 1% FCS also showed higher expression of stromal cell marker 146, PDGF receptor β and α-SMA while exhibiting similar expression levels of procollagen type I comparing with cells in 10% FCS. This suggests that 1% FCS cultured cells were also in an earlier stage than 10% FCS cultured cells, but in a later stage compared to N2B27 cultured cell implied by their expression of α-SMA and procollagen type I (Fig. 4). These results shows that reduced serum and non-serum condition both have the capacity to maintain a cell with an earlier phenotype and that FCS drives the differentiation process to a more mature phenotype. This inhibitory effect was more pronounced in these early cultures compared to passage 2 and 8 cells suggesting that the initiation of the differentiation process occurs early on in these primary cultures. Thus early passage cells must be used in future experiments.

PDGF-BB is a mitogen for mesenchymal cells in vitro and has been suggested to be involved in pericyte recruitment to blood vessels during the final stages of angiogenesis (Furuhashi et al., 2004). The TGF-β superfamily is generally considered as promoter of mesenchymal cell differentiation and scar formation (Chegini, 1997; Laiho and Kesk-Oja, 1992). In the present
investigation neither PDGF-BB nor TGF-β had an influence in 7 and 14 day cultures. However, at 21 days certain differences had begun to emerge (Fig. 5). Again stimulation was performed on passage 2 cells that had already differentiated. To gain support for this, one ongoing experiment is to separate earlier subpopulations of cd146 positive cells from passage 2 cells by FACS, and then stimulate both cd146 positive and cd146 negative subpopulations and investigate the effects of these two growth factors. Another experiment is to perform the stimulation on cells immediately following isolation from the placenta. This will require more large-scale isolations from individual placentas may need to obtain adequate numbers of lower passage cells.

Emerging cells from placenta MVFs have been shown to be capable to differentiate into collagen type I-producing cells (Ivarsson et al., 1996). Based on the marker expression profile, we divided the differentiation process into three stages. The differential expression of these markers offers us a way to isolate cells in these different stages (subpopulations) from crude cells. Subpopulation 1 expresses both stro1 and cd146 (mesenchymal stem cells). Subpopulation 2 cells are cd146 positive but stro1 negative (pericytes). Subpopulation 3 cells are negative for both stro1 and cd146 (fibroblast). Thus, according to our hypothesis subpopulation 3 is supposed to produce collagen type I, while subpopulation 2 is supposed to produce much less collagen type I. When procollagen type 1 protein levels were determined in western blots, subpopulation 3 expressed much higher levels compared to subpopulation 2 of a protein of the predicted molecular weight. This further supports that our cell separation protocols distinguish between pericytes and collagen type I-producing fibroblasts. However, the tumor epithelial cell line, which is supposed to work as negative control, also gave strong signal. Normally, epithelial cell should not produce collagen type I. This may be an in vitro artifact or that these epithelial cells have undergone EMT or epithelial-mesenchymal transition. Further experiments will be needed using different cell lines to shed light on this issue (Fig. 6).

In this project, MVFs were isolated from human placenta, and mesenchymal stem cells were obtained. In culture these cells differentiated into collagen type I producing fibroblasts via the microvascular pericyte. Experiments with the purpose of stabilizing the three different phenotypes were partly successful. Earlier stage or passage of cells and other non-serum medium type should be tried in the future for pericytes maintenance. Once a culture system where the pericyte phenotype can be stabilized and maintained in depth studies of this cell types function in vitro can be started.
Materials and Methods

Isolation of pericytes from human placenta

Newly delivered full-term placentas were obtained from the Maternity Unit at Uppsala Academic Hospital. Small pieces from the chorion villi were cut out. Excess blood was washed away by injection of PBS followed with three times washing in PBS. The tissue pieces were minced to small pieces in a table meat mincer (mid-tough settings). The minced pieces were washed three times in PBS by 5 minutes 200 g centrifugation, then dissociated in 5 mg/ml collagenase type H (SigmaAldrich) in buffer B (70 mM NaCl, 7 mM KCl, 5 mM CaCl₂, 100 mM HEPES, pH 7.6, 1,5% bovine serum albumin (BSA)) on a shaker for 2 h at 37°C. The digest was filtered through a metal filter to remove debris and larger vessel fragments. Filtrate was centrifuged at 200 g for 5 minutes in PBS. Red blood cells were removed by discarding the pellet. This step was repeated three times. The pellet containing microvascular fragments (MVF) was diluted in RPMI (Gibco) containing 10% FCS (Biowest), 1% 1 M Hepes, 2.5 μg/μl Amphotericin (Sigma-Aldrich), and 50 μg/ml Pest (penicillin G, streptomycin-sulfate), and plated on T-25 culture flasks coated with 50 μg/ml bovine collagen (Vitrogen, CohesionTech).

Culture of MVFs from human placenta

After the newly isolated MVFs attached to the cell culture flasks, the medium were changed so that the MVFs from one placenta were cultivated separately in: 1) RPMI (GibcoBRL) containing 10% FCS (Biowest), 1% 1M Hepes, 2.5 μg/μl Amphotericin (Sigma-Aldrich), and 50 μg/ml Pest (penicillin G, streptomycin-sulfate), 2) RPMI (GibcoBRL) containing 1% FCS (Biowest), the same concentration of Hepes and antibiotics 3) N2B27 (DMEM/F12 (Invitrogen) and Neurobasal (Invitrogen) (V:V=1:1) containing 25 μg/ml insulin, 100 μg/ml apo-transferrin, 6 ng/ml progesterone, 16 μg/ml putrescine, 30 nM sodium selenite, 50 μg/ml BSA, 1% B27, 1mM L-glutamine, 7 × 10⁻⁵(v/v) β-mercaptoethanol) and the same concentration of antibiotics.

Antibodies

The primary antibodies, their sources and specificities are CD31 (DAKO), PAL-E (Sanbios, Netherlands), Desmin, (DAKO clone D33), smooth muscle α-actin (Sigma, mAb clone 1A4), PDGF β-receptor (provided by Dr. K. Rubin, MAb Clone PDGFR-β), procollagen type 1 c-propeptide, cd146 (abcam), prolyl-4-hydroxylase (DAKO mAb clone 5B5), Cytokeratin (Sigma), FSP (Sigma). The polyclonal antibody (Pab) recognizing NG2 was from Chemicon, Temcula, CA.

Normal rabbit, mouse, and horse serum; rat, rabbit, and mouse IgG were purchased from Sigma. The biotinylated horse anti-mouse antibody, the rabbit anti-rat Texas Red avidin D conjugate, fluorescein-conjugated goat anti-mouse IgM, fluorescein-conjugated rabbit anti-rat IgG, allophycocyanin-conjugated goat anti-mouse IgG, fluorescein-conjugated goat anti-rabbit IgG, horse anti-mouse IgG, goat anti-rabbit IgG Texas Red conjugate were purchased from Vector Laboratories (Burlingame, CA). Fluorescein-conjugated goat anti-mouse IgG was purchased from BD and the biotinylated swine anti-rabbit (Fab2 from DAKO (Carpinteria, CA). Phalliodin Texas Red conjugate was from Invitrogen.
**Immunohistochemistry staining of cells**

Ten thousands pericytes were seeded on bovine collagen type I coated coverslips (50 µg/ml) in 24 well plates in RPMI (GibcoBRL) containing 10% FCS and antibiotics at least one day before use. Cells were washed three times in PBS and then fixed in 4% paraformaldehyde or 70% methanol for 10 minutes at room temperature. Coverslips were then rinsed three times in PBS, permeabilized in PBS with 0.2% Tween for 20 minutes, rinsed three times in PBS, and then blocked in PBS with 0.5% BSA and 0.1 M glycine, pH7.3. Coverslips were incubated with primary antibody for 1 h, rinsed, then incubated with the appropriate biotinylated secondary antibody, and rinsed. Color was developed with the Vectastain ABC elite kit (Vector Laboratories) with diaminobenzidine as the peroxidase substrate. Coverslips were then mounted in crystal mount (Biomed, Foster City, CA).

**Immunofluorescent staining of cells**

Cells were grown, fixed, permeabilized and incubated with primary antibody in the same way as immunohistochemical cell staining. Coverslips were incubated with appropriate fluorescent conjugated secondary antibody and rinsed. Coverslips were then mounted in Fluoromount G (Biowest).

**Cell culture in 1%, 10% serum and serum free conditions**

Passage 2 cells isolated from the same placenta were seeded on bovine collagen type I (50 µg/ml) coated 6-well plates (5×10⁴ cells/well). They were cultured separately in RPMI (GibcoBRL) containing 1% or 10% FCS or N2B27 with antibiotics. After 3 and 7 days cells were harvested and examined by flow cytometry.

**PDGF-BB and TGF-β stimulation**

Passage 2 pericytes isolated from the same placenta were seeded on bovine collagen type I (50 µg/ml) coated 6-well plates (5×10⁴ cells/well). They were cultivated separately in 1) RPMI (GibcoBRL) containing 10% FCS, 2) RPMI (GibcoBRL) containing 1% FCS, and 3) N2B27 with antibiotics. For each type of media, three conditions were set: medium containing 1) PDGF-BB (20 ng/ml) 2) TGF-β (10 ng/ml) and 3) without external growth factors. After 3, 7 and 14 days, cells were harvested, and protein expression was examined via flow cytometry.

**Flow cytometry**

Cells were detached by addition of 2-3 ml trypsin-EDTA (SVA) for 5-10 minutes at 37°C. Trypsin was neutralized with RPMI (Gibco) containing 10% FCS. Cells were centrifuged at 200 g for 10 minutes and collected in the pellet. If an internal marker was to be used, cells were fixed in 4% paraformaldehyde for 10 minutes on ice, washed in FACS buffer (PBS containing 0.1% FCS) and pelleted by 5 minutes 200 g centrifugation in cold room, then permeabilized with 0.2% saponin for 10 minutes on ice and then washed in FACS buffer. Cells were incubated with primary antibody on ice for 20 minutes before washed with FACS buffer. Secondary antibody was added to the cells for 20 minutes on ice, and then washed with FACS buffer. All the antibodies were diluted in FACS buffer. Cells were then
resuspended in 300µl PBS and transferred to a Beckton Dickson FACS tube. Flow cytometry was performed on a FACScan™ (Becton Dickson) flow cytometer and analyzed with the software CellQuest™ (Becton Dickson).

**Fluorescence-activated cell sorting (FACS)**

Cells were labeled in the same way as described above. After labeling, 200µl trypsin-EDTA was added to the cells for 3 minutes before neutralization by serum and washed away. Cells were then resuspended in PBS at 3 million cells/600µl and transferred to Becton Dickinson FACS tubes. FACS sorting was performed on a FACScan™ (Becton Dickinson) flow cytometer and analyzed with the software CellQuest™ (Becton Dickinson). Sorted cells were kept on ice before 10 minutes centrifugation at 200 g. Cells were then collected for further use.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis**

Samples containing equal amount of protein were loaded onto 6% polyacrylamide gel: the The stocking gel (5%) containing 5.1% acrylamide, 13% of 1 M Tris-HCl pH6.8, 0.1%SDS, 0.1% APS, 0.1% TEMED, 68% of distilled water was layered ontop of the separation gel containing 6% acrylamide, 26% of 1.5 M Tris-HCl pH8.8, 0.1% SDS, 0.1% APS (ammonium persulfate), 0.08% of TEMED (N,N,N’,N’-tetramethylethylendiamine) 52% distilled water. Samples were electrophoresed with buffer containing 2.85% (w/v) Glycine, 0.6% (w/v) Tris and 0.1% SDS at 100 volts. The samples on the gel were then transferred to nitrocellulose membranes (Amersham) by semi-dry electrical transferring machine (BIO-RAD SD, SEMI-DRY TRANSFER CELL) at 15 volts for 45 minutes. The membranes were blocked with 5% BSA in TBS with 0.2% azid overnight at 4°C. The membranes were washed twice in TBS with 5% BSA and 0.2% Tween then probed with goat anti-procollagen type I antibody (Santa Cruz) for 1.5 h at room temperature. The primary antibody was used at 1:200 dilution in TBS with 5% BSA and 0.1% Tween. The membranes were washed twice to remove unbound antibodies. Then membranes were incubated with secondary HRP conjugated donkey anti-goat antibody (Santa Cruz) at 1:5000 dilution for 1.5 h at room temperature. Membranes were washed six times for 10 minutes, and then incubated for 20 seconds in ECL reagent solution containing 1.25 mM luminol (5-amino-2,3-dihydro-1,4-ptalazinedione) in 0.1 M Tris-HCl pH8.5 with 1% of 68 mM p-Coumaric acid in DMSO (Sigma) and 0.09% H₂O₂.
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