Isoform-Specific Regulation of the Actin-Organizing Protein Palladin during TGF-β1-Induced Myofibroblast Differentiation

Mikko J. Rönty[1], Suvi-Katri Leivonen[2], Boris Hinz[3], Andrew Rachlin[4], Carol A. Otey[4], Veli-Matti Kähäri[2] and Olli M. Carpén[1,5]

Contractile myofibroblasts are responsible for remodeling of extracellular matrix during wound healing; however, their continued activity results in various fibrocontractive diseases. Conversion of fibroblasts into myofibroblasts is induced by transforming growth factor-β1 (TGF-β1) and is hallmarked by the neo-expression of α-smooth muscle actin (α-SMA), a commonly used myofibroblast marker. Moreover, myofibroblast differentiation and acquisition of the contractile phenotype involves functionally important alterations in the expression of actin-organizing proteins. We investigated whether myofibroblast differentiation is accompanied by changes in the expression of palladin, a cytoskeletal protein that controls stress fiber integrity. Palladin is expressed as several isoforms, including major 3Ig (90 kDa) and 4Ig (140 kDa) forms that differ in their N-terminal sequence. Expression of the 4Ig isoform is strongly induced in fibroblast stress fibers upon TGF-β1 treatment preceding α-SMA upregulation. TGF-β1 induced upregulation of palladin is mediated both by Smad and mitogen-activated protein kinase pathways. Furthermore, palladin 4Ig-isoform is co-expressed with α-SMA *in vivo* in experimental rat wounds and in human myofibroblast-containing lesions. Taken together these results identify palladin 4Ig as a novel marker of myofibroblast conversion *in vitro* and *in vivo*. They also provide for the first time information about the signaling cascades involved in the regulation of palladin expression.

Journal of Investigative Dermatology (2006) **126,** 2387–2396. doi:10.1038/sj.jid.5700427; published online 22 June 2006

INTRODUCTION

Myofibroblasts are specialized cells that originate from fibroblasts during both physiological and pathological processes. Myofibroblasts secrete extracellular proteins, proteases, cytokines, and growth factors and actively participate in modulation of the extracellular matrix (ECM) during wound healing (Powell *et al.*, 1999; Tomasek *et al.*, 2002). The cells account for the contractile potential of granulation tissue, which ensures the rapid and efficient closure of dermal wounds (Hinz and Gabbiani, 2003b). After wound closure myofibroblasts usually disappear by apoptosis (Desmouliere and Gabbiani, 1995). However, in fibrocontractive diseases, for example hypertrophic scars, liver cirrhosis, idiopathic lung fibrosis, and glomerulosclerosis, myofibroblasts persist and contribute to disease progress by overproduction of ECM components and by excessive contraction (Desmouliere *et al*, 2003; Gabbiani, 2003). They are also a key element in the stromal reaction induced by invasive neoplastic cells (Desmouliere *et al.*, 2004).

Differentiation of myofibroblasts from normal fibroblasts involves both mechanical stimulus to the cells by tensile forces in the ECM and chemical stimuli by growth factors (Tomasek *et al.*, 2002). During this process, myofibroblasts first become proto-myofibroblasts and, upon maturation, acquire smooth muscle characteristics, including changes in the structure of contractile actin cytoskeleton and concomitant neo-expression of actin-associated proteins (Malmstrom *et al.*, 2004). One of the neo-expressed proteins is α-smooth muscle actin (α-SMA). α-SMA is considered as the most reliable marker of myofibroblastic differentiation and it is widely used both in experimental models and diagnostic pathology (Darby *et al.*, 1990). Besides α-SMA, myofibroblasts can express smooth muscle myosin heavy chains and calponin, both of which are involved in the contractile activity of the cells (Tomasek *et al.*, 2002).

[1]*Neuroscience Program and Department of Pathology, University of Helsinki and Helsinki University Central Hospital, Helsinki, Finland;* [2]*Department of Dermatology, Medical Biochemistry and Molecular Biology, and MediCity Research Laboratory, University of Turku, Turku, Finland;* [3]*Laboratory of Cell Biophysics (LCB), École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland;* [4]*Department of Cell and Molecular Physiology, University of North Carolina, Chapel Hill, North Carolina, USA and* [5]*Department of Pathology, University of Turku and Turku University Central Hospital, Turku, Finland*

Correspondence: Dr Mikko J. Rönty, Department of Pathology, PO Box 21, University of Helsinki, Helsinki 00014, Finland.
E-mail: mikko.ronty@helsinki.fi

Abbreviations: GFP, enhanced green fluorescent protein; MAPK, mitogen-activated protein kinase; α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1

Received 4 November 2005; revised 30 March 2006; accepted 13 April 2006; published online 22 June 2006

However, myofibroblasts do not attain all the molecular characteristics of smooth muscle cells, for instance they only occasionally express desmin and do not express smoothelin (Tomasek *et al.*, 2002).

An important factor in myofibroblast conversion is transforming growth factor-β1 (TGF-β1). TGF-β1 expression is upregulated in dermal wounds, where it is released by platelets and produced by keratinocytes, inflammatory leukocytes and fibroblasts (O'Kane and Ferguson, 1997). TGF-β1 mediates its effects primarily via Smad and mitogen-activated protein kinase (MAPK) pathways (Massague, 2000; Massague and Wotton, 2000). Most importantly for myofibroblastic differentiation, TGF-β1 can induce the expression of α-SMA via Smad3 (Hu *et al.*, 2003). TGF-β1 stimulation of fibroblasts leads also to activation of ERK1/2 and p38, both of which are members of the MAPK signaling pathway (Ravanti *et al.*, 1999a, b). There is considerable cross-talk between Smad and MAPK signaling cascades, and MAPKs can either positively or negatively influence Smad activation (Derynck and Zhang, 2003).

Palladin together with myotilin and myopalladin forms a novel subfamily of cytoskeletal Ig-domain-containing proteins (Salmikangas *et al.*, 1999; Parast and Otey, 2000; Bang *et al.*, 2001; Mykkänen *et al.*, 2001). Myotilin and myopalladin are mainly expressed in striated muscle, whereas palladin is more widely expressed both in epithelial and mesenchymal cells (Parast and Otey, 2000; Mykkänen *et al.*, 2001). Palladin exists as multiple isoforms, whose transcription is regulated in a cell type-specific manner. The genomic structure of murine palladin implies the presence of three different promoter regions, which may allow the tissue-specific expression of different isoforms (Rachlin and Otey, 2006). Palladin localizes to the Z-discs in cardiomyocytes, and to stress fiber dense bodies and focal adhesions in non-muscle cells (Parast and Otey, 2000; Mykkänen *et al.*, 2001). By interacting with several actin-associated proteins including α-actinin, ezrin, VASP, ArgBP2, and profilin, palladin can serve as a scaffold that connects molecules with different functional activities (Mykkänen *et al.*, 2001; Boukhelifa *et al.*, 2004; Rönty *et al.*, 2004; Rönty *et al.*, 2005; Boukhelifa *et al*, 2006). Antisense experiments and studies with palladin knock-out fibroblasts have shown that downregulation of palladin leads to disruption of actin-containing stress fibers, whereas transient overexpression leads to re-organization of the actin cytoskeleton and induction of thick actin bundles (Parast and Otey, 2000; Boukhelifa *et al.*, 2003; Rönty *et al.*, 2004; Luo *et al.*, 2005; Rachlin and Otey, 2006). *In vivo*, loss of palladin causes embryonic lethality due to an apparent cell migration defect resulting in defective neural tube and ventral closure (Luo *et al.*, 2005).

In this study, we have analyzed palladin isoform expression during fibroblast-to-myofibroblast conversion. We show that palladin is upregulated during the differentiation process in response to TGF-β1 and that the 4Ig isoform is neo-expressed in myofibroblasts. The presence of the 4Ig isoform is also demonstrated *in vivo* in a rat wound model and in tissues rich in myofibroblasts. Finally, the TGF-β1 signaling pathways involved in the regulation are identified.

RESULTS

Characterization of isoform-specific palladin antibodies and expression of palladin isoforms

The cloning and characterization of mouse palladin has identified at least five different isoforms, including a novel transcript that contains four Ig domains (Rachlin and Otey, 2006). Western blot analysis with a C-terminal palladin antibody (Ab953) has previously demonstrated that several forms of palladin, including 90 and 140 kDa polypeptides, are expressed in U251 human glioma cells (Mykkänen *et al.*, 2001). The 140 kDa protein presumably represents the 4Ig isoform (Figure 1a) and therefore U251 mRNA was used as a template to clone the 5′-sequence extension of the human 4Ig homologue by reverse transcriptase-PCR. New antibodies against palladin sequences were raised to obtain further insight on the presence and localization of various forms of palladin. The antigen used to produce the 3Ig antibody corresponds to the previously described proline-rich N-terminal segment of the Ab023209 sequence (Mykkänen *et al.*, 2001). The novel 4Ig sequence extension was produced as a glutathione-*S*-transferase-fusion protein and used as an antigen to produce a 4Ig-specific antibody (Figure 1a). The reactivity of palladin antibodies was verified by Western blot analysis of cells transfected with various green fluorescent protein (GFP)-palladin constructs (Figure 1b). The Ab-3Ig recognized the GFP-3Ig construct and Ab-4Ig-Hu raised against the N-terminus of the human 4Ig sequence reacted with GFP-4IgN-term construct. No crossreactivity or reactivity against GFP was observed. U251 cell lysates were next probed with palladin antibodies Ab-3Ig, Ab-4Ig-Hu, Ab-4Ig-Mo, Ab953, and a mixture of palladin mAbs (4D10, 7C6, 9C12, and 1E6) (Parast and Otey, 2000). As expected, the antibodies reacted with several protein bands (Figure 1c). One of the bands (140 kDa) was recognized by all antibodies verifying that the 4Ig sequence is indeed expressed. The previously described 3Ig (90 kDa) major isoform (Parast and Otey, 2000; Mykkänen *et al.*, 2001) was recognized by Ab-3Ig, Ab953, and the mAb but not by either 4Ig antibody. Thus, it can be concluded that Ab-3Ig reacts both with the 3Ig and 4Ig isoforms, whereas the 4Ig antibodies react with the 4Ig but not the 3Ig isoform. In U251 cells additional bands migrating at 115, 70, and 50 kDa were also detected. Similar sized bands have been detected in previous reports with independent antibodies as well as antibodies used in this study (Parast and Otey, 2000; Bang *et al.*, 2001; Mykkänen *et al.*, 2001; Luo *et al.*, 2005). These bands could present additional differentially spliced transcripts. In immunostaining of U251 cells (Figure 1d), the 4Ig antibodies showed a similar staining as Ab-3Ig and Ab953, decorating mainly stress fiber dense bodies and focal adhesions.

Regulation of the isoform-specific expression of palladin during myofibroblastic modulation

Next, we studied the expression and subcellular localization of different palladin isoforms in fibroblasts during the TGF-β1 induced myofibroblastic differentiation. Under basal culture conditions Ab-3Ig demonstrated an analogous staining pattern in human skin fibroblasts as in U251 cells, whereas

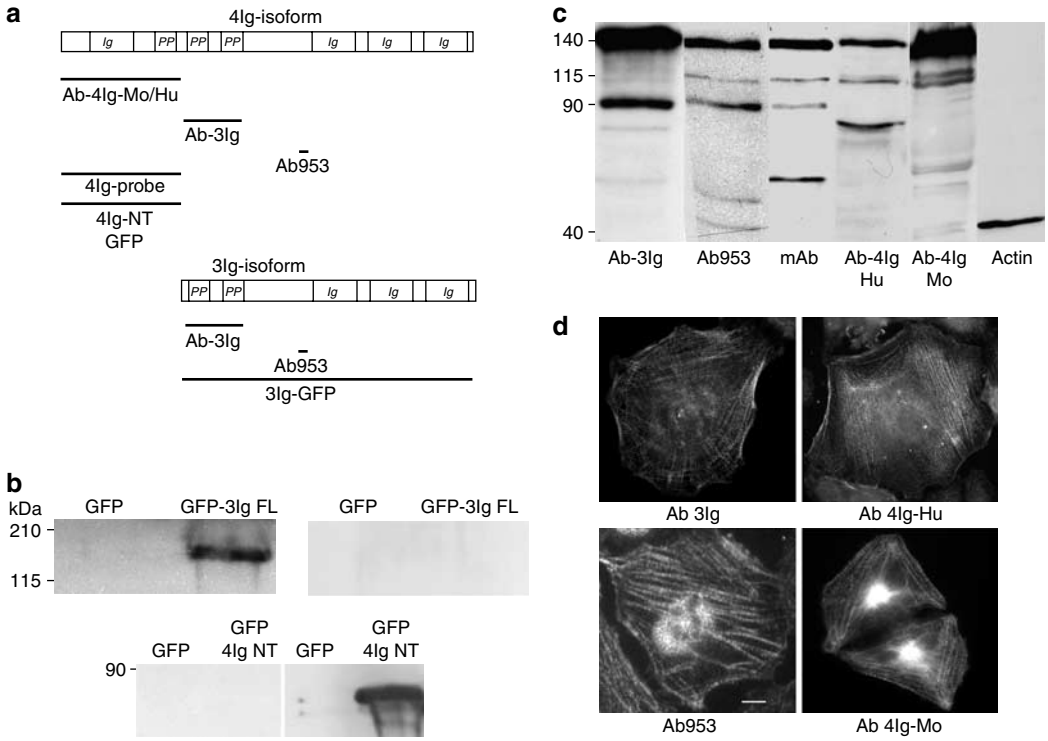

Figure 1. Characterization of the palladin antibodies. (**a**) A schematic illustration of the palladin isoforms and the regions used for recombinant protein/antibody production, GFP-constructs and 4Ig Northern blot probe. (PP = poly-proline, Ig = IgC2 domain). (**b**) COS-7 cells were transfected with the indicated GFP-palladin constructs and the lysates were blotted with antibodies Ab-3Ig (left side) and Ab-4Ig-Hu (right side). In the upper panel COS-7 cells were transfected with a full-length 3Ig isoform construct. Ab-3Ig recognizes the transfected protein whereas Ab-4Ig-Hu does not. In the lower panel, COS-7 cells were transfected with a construct that encodes the N-terminal sequence specific for 4Ig isoform. Ab-4Ig-Hu recognizes the transfected construct while Ab-3Ig does not. Neither antibody reacted with GFP. (**c**) U251 cell lysates were blotted with palladin antibodies. All antibodies recognize the 140 kDa 4Ig isoform. Ab-3Ig, Ab953, and the mAb mixture also recognize the 90 kDa 3Ig isoform, whereas Ab-4Ig-Hu and Mo do not. None of the palladin antibodies react with β-actin, which is shown as a control. (**d**) U251 cells were stained with different palladin antibodies. All antibodies show a similar punctate staining pattern along the stress fibers corresponding to dense regions, and in some focal adhesions. Bar = 5 μm.

Ab-4Ig-Hu showed no specific reactivity. In cells treated with 5 ng/ml of TGF-β1 for 12–96 hours, a more robust stress fiber staining was observed with Ab-3Ig. After treatment, also Ab-4Ig-Hu showed a strong punctate stress fiber staining indicating that TGF-β1 induces expression of the 4Ig isoform. The changes in palladin expression were similar in the presence and absence of FCS. In line with previous studies, SMA was also upregulated during the myofibroblastic differentiation and it colocalized with palladin in stress fibers (Figure 2a–c). A similar result could be seen in Western blot experiments, where TGF-β1 treatment resulted in upregulation of the 90 kDa 3Ig isoform and neo-expression of the 140 kDa 4Ig isoform (Figure 2d). Upregulation of the 4Ig isoform was rapid and high levels were present already after 24 hours of TGF-β1 treatment. Induction of α-SMA expression occurred later and was markedly upregulated only after 48 hours of treatment. Thus, palladin 4Ig expression seems to precede α-SMA.

The TGF-β1-induced palladin expression is mediated by Smad3, p38, and ERK1/2

We elucidated the role of Smad signaling in TGF-β1-induced palladin expression. We utilized adenoviral gene delivery of wild-type Smad3 (RAdSmad3) to obtain overexpression of Smad3, and RAd dominant-negative Smad3 (Smad3DN), and inhibitory Smad7 (RAdSmad7) to inhibit Smad signaling. Human gingival fibroblasts were infected with the corresponding Smad adenoviruses and empty control adenovirus (RAdpCA3) at multiplicity of infection 500, and treated with TGF-β1 (5 ng/ml) for 24 hours, as indicated. As shown in Figure 3a, a 24-hour TGF-β1 stimulation markedly enhanced palladin expression in RAdpCA3-infected cells. Overexpression of Smad3 further enhanced the TGF-β1-elicited palladin expression. Conversely, inhibition of Smad signaling by adenoviral expression of Smad3DN and Smad7 markedly inhibited the TGF-β1-induced levels of palladin (Figure 3a). TGF-β1 stimulation also enhanced the expression of SMA, and this was inhibited by adenoviral expression of Smad3DN and Smad7 (Figure 3a). Together, these results imply that Smad signaling, in particular via Smad3, mediates the TGF-β1-induced expression of palladin and α-SMA in fibroblasts.

TGF-β1 activates ERK1/2 and p38 MAPK pathways in gingival fibroblasts (Ravanti *et al.*, 1999a). In addition, Smad3 is involved in regulating the TGF-β1-elicited expression of matrix metalloproteinase-13 in cooperation with p38 MAPK (Leivonen *et al.*, 2002), and the TGF-β1-elicited expression of

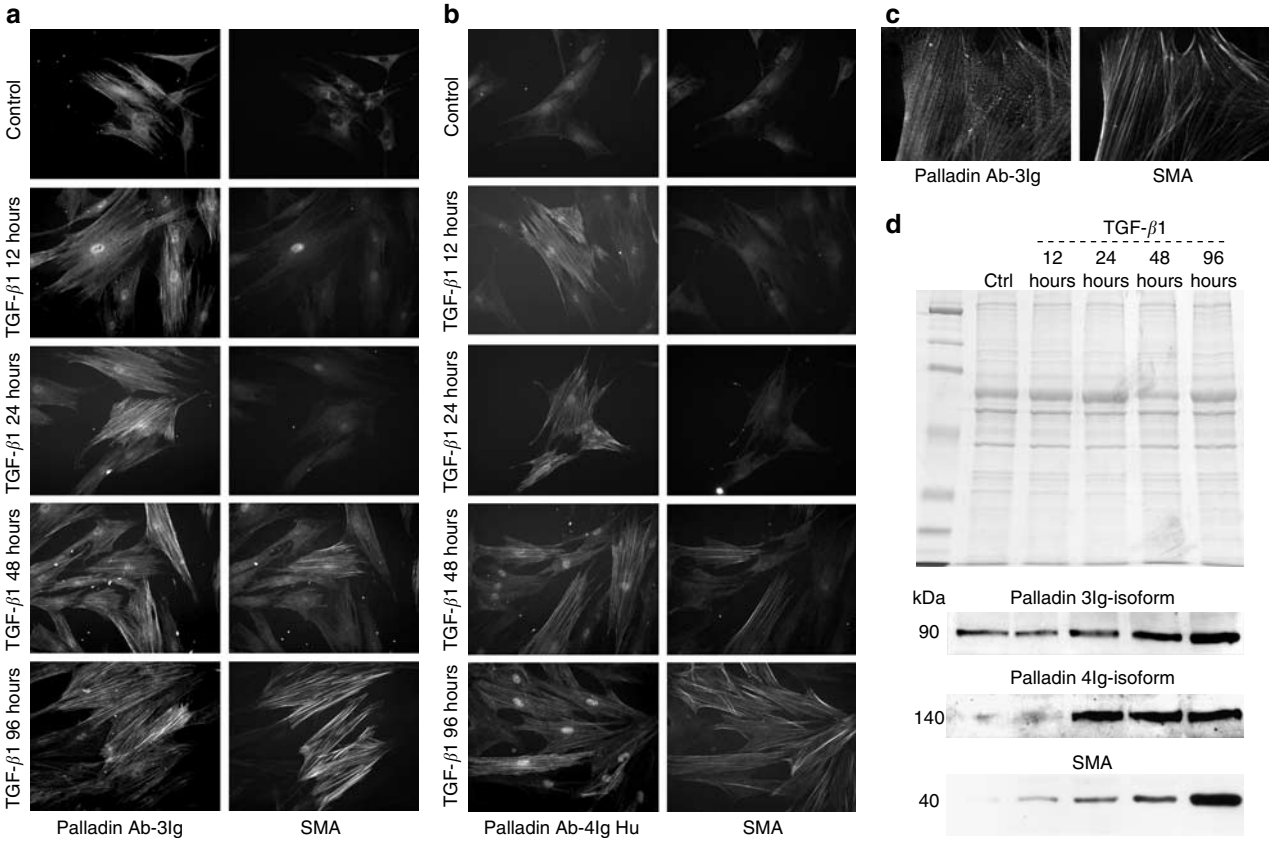

Figure 2. Immunoreactivity of fibroblasts with palladin and α-SMA antibodies before and after treatment with TGF-β1. Human dermal fibroblasts were grown on coverslips and treated with TGF-β1 (5 ng/ml) for 12–96 hours. Subsequently, the cells were fixed and stained. (**a**) Ab-3Ig, which recognizes both the 3Ig and 4Ig isoforms show a punctate staining pattern along the stress fibers both in the control cells and TGF-β1-treated cells. However, the staining is more robust after 12 hours of treatment. (**b**) Ab-4Ig-Hu, which recognizes only the 4Ig isoform shows only weak diffuse staining in untreated cells. After 12 hours of TGF-β1 treatment a signal similar to Ab-3Ig is seen along the stress fibers. TGF-β1 treatment induces also expression of α-SMA, but the signal is evident only after 48 hours. After 96 hours most of the cells are positive for both 4Ig palladin and α-SMA (**a** and **b**). (**c**) At a higher magnification the punctate staining pattern of palladin along α-SMA-positive stress fibers is evident. The expression of palladin 3Ig and 4Ig isoforms together with α-SMA was also studied by Western blotting. Palladin 3Ig is expressed already in the control cells but the expression increases during TGF-β1treatment. Palladin 4Ig is neo-expressed in fibroblasts. Palladin isoforms are upregulated prior to α-SMA appearance. (**d**) A Coomassie-stained gel is shown as a loading control.

CTGF in cooperation with ERK1/2 (Leivonen *et al.*, 2005). Therefore, we first examined whether ERK1/2 and p38 MAPK pathways play a role in mediating the effects of TGF-β1 on palladin gene expression in human gingival fibroblasts. We used PD98059, an inhibitor for MEK1, the upstream activator of ERK1/2, and SB203580, a specific chemical inhibitor for p38 MAPK. Both PD98059 and SB203580 potently down-regulated TGF-β1-induced palladin expression at the protein level (Figure 3b), indicating that p38 and ERK1/2 MAPK signaling cascades are important for mediating the effects of TGF-β1 on palladin gene expression. Inhibition of the p38 pathway by SB203580 also reduced the TGF-β1-induced α-SMA expression, and co-treatment of fibroblasts with PD98059 and SB203580 abolished α-SMA expression (Figure 3b).

We examined the possible cross-talk between the MAPK and Smad3 pathways in mediating the TGF-β1-elicited expression of palladin. Human gingival fibroblasts were infected with recombinant adenoviruses for Smad3 and Smad4 together with adenoviruses for wild-type p38α (RAdp38α), and constitutively active MKK3b (RAdMKK3bE), an upstream activator of p38, and incubated for 24 hours. In the absence of TGF-β1, activation of p38 by a constitutively active mutant of its upstream activator MKK3b induced the expression of palladin 4Ig mRNA (Figure 3c). Overexpression of Smad3 further enhanced the upregulatory effect of MKK3bE on palladin mRNA expression. Next, gingival fibroblasts were infected with adenoviruses for constitutively active MEK1 (RAdMEK1CA) alone or in combination with RAdSmad3 and RAdMKK3bE. Activation of ERK1/2 with co-expression of Smad3 resulted in marked induction in palladin 4Ig isoform expression (Figure 3d). This effect was further enhanced by co-expression of constitutively active MKK3b. Together, these results imply that interplay between ERK1/2, p38, and Smad3 signaling plays an important role in regulating palladin gene expression.

Palladin is expressed in experimental rat dermal wounds

Next, we studied the expression of palladin in experimental rat dermal wounds. Sections of 6 and 9-day-old granulation

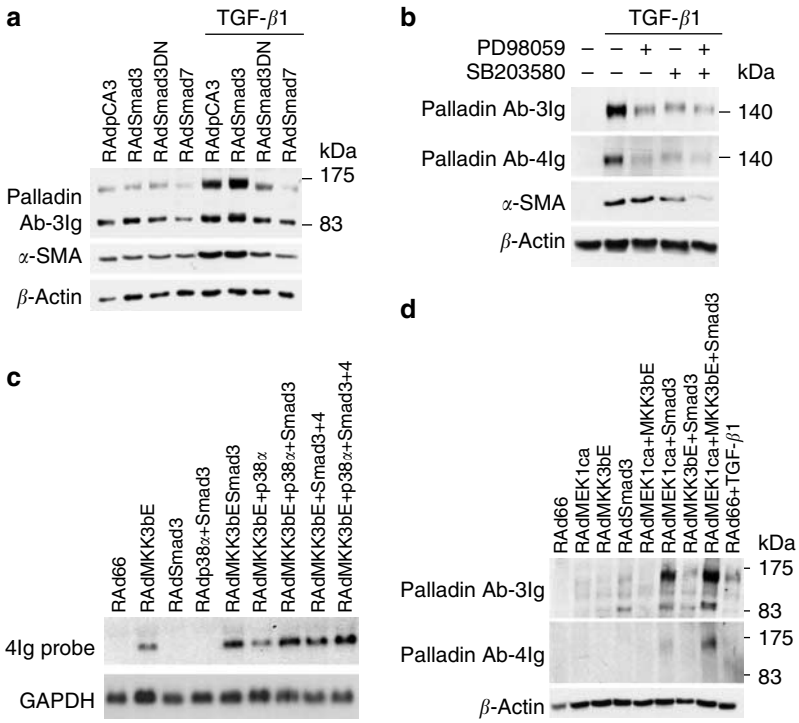

Figure 3. Palladin gene expression in fibroblasts is regulated by Smad3, ERK1/2, and p38 signaling. (**a**) Normal human gingival fibroblasts were infected with adenoviruses for RAdSmad3, RAdSmad3DN, Smad7 (RAdSmad7), or with empty control virus RAdpCA3 at multiplicity of infection 500, and incubated for 24 hours. Thereafter, the cells were treated with TGF-β1 for 24 hours. Cell lysates were analyzed for the expression of palladin 4Ig isoform by Western blotting. Equal loading was confirmed by stripping and reprobing the same filter with β-actin. The effect of TGF-β1 on the palladin expression was further augmented by wild-type Smad3 construct and it could be inhibited by either Smad3DN or by an inhibitory Smad7 expression. (**b**) Human gingival fibroblasts were serum starved for 18 hours, and treated for 1 hour with PD98059 (30 μM), or SB203580 (10 μM), inhibitors for MEK1 or p38, respectively. Subsequently, TGF-β1 (5 ng/ml) was added for additional 16 hours. The cells were lysed and analyzed for palladin 4Ig and α-SMA expression. The effect of TGF-β1 on the 4Ig expression is inhibited by MAPK inhibitors. Both the p38 inhibitor SB203580 and the MEK1/2 inhibitor PD98059 inhibited the upregulation individually and when combined the effect was even more pronounced. Similar result could be appreciated with α-SMA expression. (**c**) Human gingival fibroblasts were infected with recombinant adenoviruses for wild-type p38α (RAdp38α), constitutively active MKK3b (RAdMKK3bE), Smad3 (RAdSmad3), Smad4 (RAdSmad4), or with empty control virus RAd66 at multiplicity of infection 500, and incubated for 24 hours. Total cellular RNA was analyzed with Northern blot hybridizations for the expression of palladin 4Ig and GAPDH mRNAs. Northern blot analysis of 4Ig palladin expression in human fibroblasts infected with adenoviruses encoding several MAPK and Smad constructs. MKK3b expression upregulates the 4Ig transcript and the effect is enhanced by co-expression of wild-type Smad3/4 constructs. (**d**) Human gingival fibroblasts were infected with recombinant adenoviruses for constitutively active MEK1 (RAdMEK1CA), constitutively active MKK3b (RAdMKK3bE), Smad3 (RAdSmad3), and control virus RAd66 as in (**c**). The cell lysates were analyzed for the expression of palladin with Western blotting. In line with mRNA analysis, active MEK1, MKK3b, and wild-type Smad3 induce expression of the palladin 4Ig isoform at protein level.

tissue from full thickness rat dermal wounds were co-stained for α-SMA, palladin 4Ig (Ab-4Ig-Mo) and palladin 3Ig (Figure 4). Both palladin antibodies stain the fibroblasts already in 6-day-old tissue when they are still negative for α-SMA and thus represent the proto-myofibroblast phenotype (Figure 4a and c). After 9 days wound healing the proto-myofibroblasts evolve into differentiated myofibroblasts by *de novo* expressing α-SMA (Figure 4b and d), palladin colocalizes with α-SMA in the stress fibers (Figure 4b and d insets).

Experiments with cultured fibroblasts showed that the palladin 3Ig isoform is expressed already at basal culture conditions whereas palladin 4Ig is neo-expressed when the cells differentiate into myofibroblasts. Therefore, we wanted to study the expression of the 4Ig isoform in more detail in the rat dermal wound model. As shown in Figure 5a, palladin 4Ig isoform is not expressed in fibroblasts of early (3 days) wound granulation tissue, which do not exhibit microfilament bundles. However, around day 6 post-wounding granulation tissue fibroblasts *de novo* express palladin 4Ig isoform in conjunction with the development of stress fibers (Figure 5b and e). Until this stage myofibroblasts are negative for α-SMA (Figure 5g–h), which is upregulated after 9 days wound healing (Figure 5c, i, and l). At both differentiation stages, palladin 4Ig isoform exhibits punctate staining pattern along stress fibers (Figure 5i inset). Arrowheads mark blood vessels, which are positive for actin and α-SMA but negative for palladin 4Ig.

Analysis of palladin expression *in vivo* in human tissue samples

The *in vivo* correlation of the *in vitro* experimental results was also studied by immunohistochemistry of human tissue specimens of conditions associated with myofibroblastic differentiation. Such conditions include fresh scar formation and the reactive proliferative lesion, nodular fasciitis (Figure

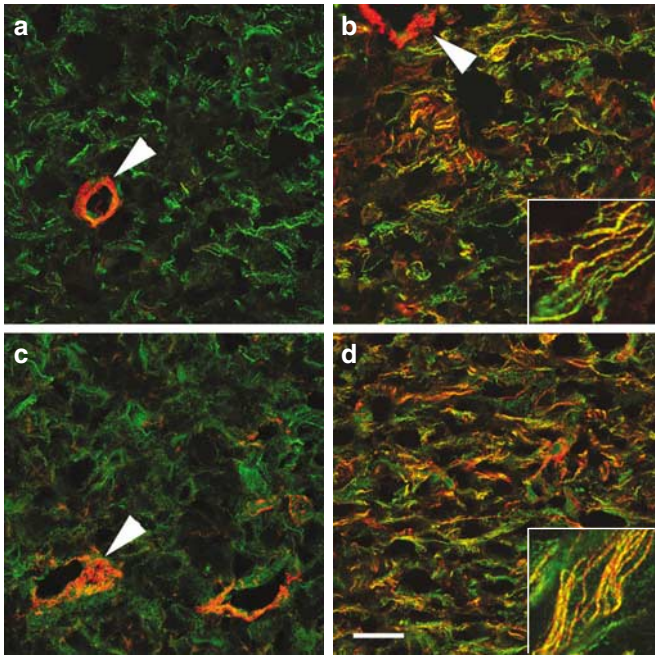

Figure 4. Palladin expression in wound granulation tissue. (**a**–**d**) Sections of (**a** and **c**) 6-day- and (**b** and **d**) 9-day-old granulation tissue from full thickness rat wounds were co-stained for α-SMA (red), (**a** and **b**, green) palladin 4Ig (Ab-4Ig-Mo) (**c** and **d**, green) and palladin 3Ig. Small vessels that are positive for α-SMA but not palladin are highlighted with arrowheads. Bar = 20 ìm. Images are representative for three independently collected series. Note that the expression of palladin 4Ig and 3Ig in 6-day-old tissue precedes myofibroblast differentiation and α-SMA expression, occurring around day 9 after wounding.

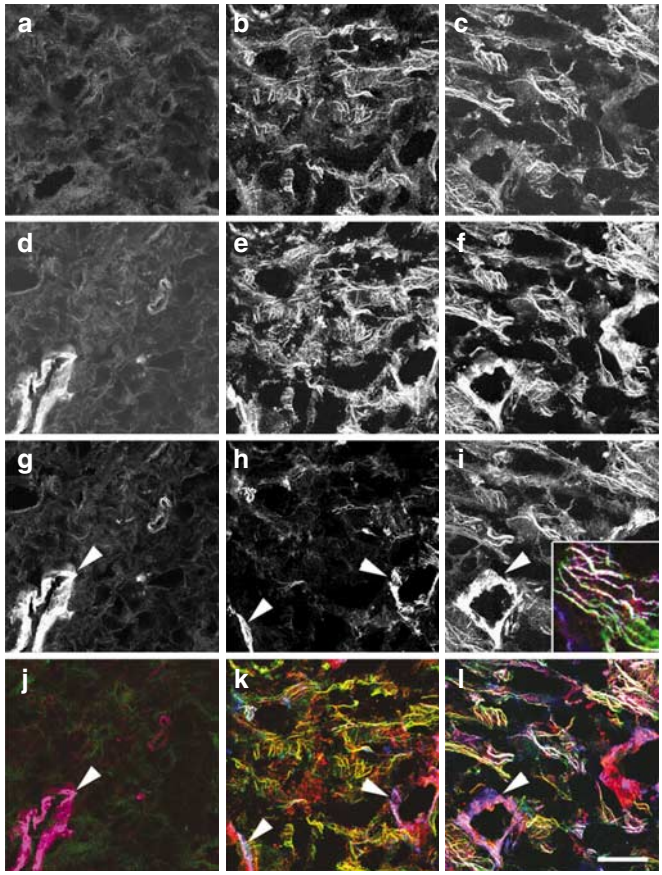

Figure 5. Palladin 4Ig expression in wound granulation tissue myofibroblasts. (**a**–**l**) Sections of (**a**, **d**, **g**, and **j**) 3-day-, (**b**, **e**, **h**, and **k**) 6-day-, and (**c**, **f**, **i**, and **l**) 9-day-old granulation tissue from full thickness rat wounds were triple stained for palladin 4Ig (Ab-4Ig-Mo) (**a**-**c**), F-actin (**d**–**f**) and α-SMA (**g**–**i**). (**j**–**k**) Overlay of staining for palladin 4Ig (green), F-actin (red), and α-SMA (blue). Bar = 20 ìm (full image) and 4 ìm (inset); images are representative for three independently collected series. Palladin 4Ig expression in granulation tissue is initiated 6 days post-wounding together with the differentiation of fibroblasts into F-actin-positive but α-SMA-negative proto-myofibroblasts.

6a and b), both of which contain a high number of myofibroblasts, and a myofibroblastic tumor of the breast (not shown). Subsequent sections of each entity were stained for α-SMA, desmin, and with two palladin antibodies; Ab-4Ig-Mo and Ab-3Ig. In all of the analyzed specimens a clear correlation between α-SMA and palladin staining could be detected, whereas desmin (a smooth muscle marker) did not stain myofibroblasts. In normal dermis the fibroblasts did not express palladin, desmin, or α-SMA (Figure 5c).

DISCUSSION

We here show that the expression of palladin isoforms is regulated in fibroblastic cells during TGF-β1-induced myofibroblast differentiation. TGF-β1 induces neo-expression of palladin 4Ig isoform and upregulates expression of the 3Ig isoform in cultured fibroblasts via Smad and MAPK pathways. The 4Ig isoform is also expressed in myofibroblastic lesions and its expression precedes upregulation of α-SMA.

TGF-β1 regulates the expression of several cytoskeletal proteins, most importantly α-SMA, during myofibroblastic differentiation (Desmouliere *et al.*, 1993; Malmstrom *et al.*, 2004). These proteins are thought to enhance the contractility of fibroblasts and for α-SMA this effect has also been shown experimentally (Hinz *et al.*, 2001). The contractile force is generated by stress fibers composed of bundles of actin and several actin-associated proteins. In myofibroblasts the contraction of the stress fibers is thought to be regulated by the Rho-Rho kinase pathway and hence produce a slower but more durable force than by the Ca^{+}-regulated contraction of smooth muscle cells (Tomasek *et al.*, 2002; Hinz and Gabbiani, 2003b). The generated force is transduced to the ECM via focal adhesions. In myofibroblasts the size and molecular composition of the focal adhesions differs from their counterparts in normal fibroblasts. The adhesions are longer and contain additional components, such as tensin. These structures, also termed ''super-mature focal adhesions'' (Dugina *et al.*, 2001, Hinz and Gabbiani, 2003a; Hinz *et al.*, 2003), are thought to enable the transmission of higher forces to the ECM produced by the more contractile myofibroblasts. Both palladin isoforms expressed in myofibroblasts localize to stress fiber dense bodies and to focal adhesions. In these structures, they interact with an important actin crosslinking protein, α-actinin (Rönty *et al.*, 2004). Localization to these specific structures suggests that palladin could be involved in

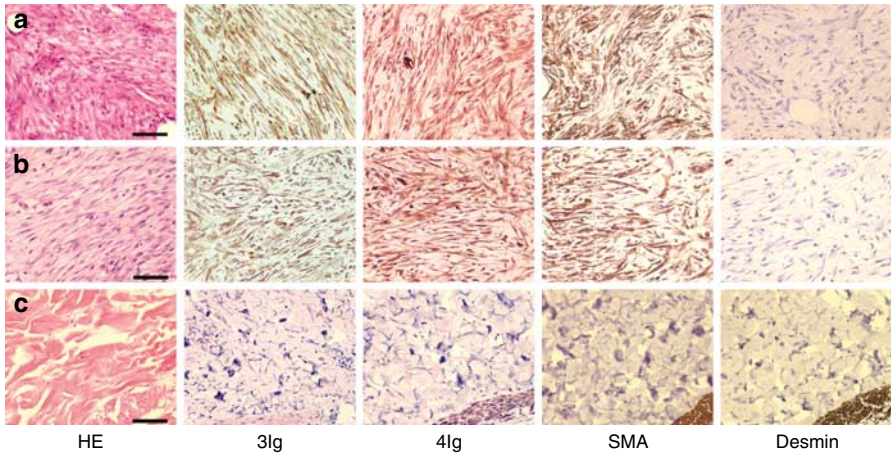

Figure 6. Immunohistochemical staining of human myofibroblastic lesions. (**a**) Fresh dermal scar, (**b**) nodular fasciitis, and (**c**) normal dermis were stained with two palladin antibodies Ab-3Ig, Ab-4Ig-Mo, and with α-SMA and desmin antibodies. In the dermal scar specimen, hematoxylin and eosin staining demonstrates reactive fibroblast-type cells within vascularized collagenous matrix. The reactive myofibroblasts show strong positivity for palladin and α-SMA, while the desmin staining is negative. Hematoxylin and eosin staining of the nodular fasciitis specimen shows that the spindle-shaped reactive cells are embedded in a loose collagenous matrix. The cells are strongly positive for both palladin and α-SMA but do not stain with desmin antibody, thus verifying their myofibroblastic nature. In the normal dermis (**c**) fibroblasts are negative for α-SMA, desmin and palladin. As a positive control for the desmin staining a small *erector pili* muscle is shown in the bottom parts of the figure. Bar = 10 μm.

the generation of contractile forces and/or force transmission to the ECM.

Palladin 4Ig isoform differs from the previously described 3Ig isoform by its N-terminus, which contains an additional IgC2 domain. In adult murine tissues, this particular isoform has been shown to be highly expressed in tissues rich in smooth muscle, such as stomach and uterus and in the cardiac muscle. The isoform shows stronger actin organizing ability than the 3Ig isoform and specifically binds to an actin-associated protein, LIM and SH3 protein 1 (LASP-1) (Rachlin and Otey, 2006). LASP-1 is homologous to nebulin, a muscle-specific protein which plays an important role in sarcomere structure and function (Tomasetto *et al.*, 1995; Chew *et al.*, 2002). Thus, palladin 4Ig isoform could be involved in TGFβ-1-induced actin remodeling and production of contractile force in myofibroblasts.

The role of TGF-β1 in myofibroblastic differentiation is widely appreciated, but the signaling pathways involved in cytoskeletal modulation are incompletely characterized. We show that TGF-β1-induced palladin expression is regulated by both the Smad and MAPK signaling pathways. The same pathways have also been shown to induce α-SMA expression during myofibroblastic differentiation (Hashimoto *et al.*, 2001b; Hu *et al.*, 2003). A direct cross-talk between the signaling cascades regulates important myofibroblast effector proteins, such as matrix metalloproteinases (Leivonen *et al.*, 2002). For palladin, active MKK3bE upregulated the 4Ig transcript when co-expressed with wild-type Smad3/4 and p38. On the protein level palladin expression was upregulated by specific activation of ERK1/2 and p38, and overexpression of Smad3. MAPK inhibitors abrogated the TGF-β1-mediated induction of 4Ig isoform expression and the effect of TGF-β1 was also blocked by expression of Smad3DN and inhibitory Smad7 constructs. These results are in line with the previously described expression of α-SMA and matrix metalloproteinases by myofibroblasts during wound healing and further support the assumption that the upregulation of palladin serves a functional role in this modulation. We have previously shown that palladin expression is also upregulated during the differentiation of dendritic cells from monocytes by IL-4 and GM-CSF (Mykkänen *et al.*, 2001). Although the cell types are different and the results are not experimentially connected, it is interesting to note that IL-4 have also been shown to upregulate the expression of SMA in human lung and synovial fibroblasts (Mattey *et al.*, 1997; Hashimoto *et al.*, 2001a). Also, GM-CSF have been shown to upregulate the number of α-SMA-expressing cells in granulation tissue, but this effect may be indirect and mediated by the recruited inflammatory leukocytes which are known to produce TGF-β1 (Rubbia-Brandt *et al.*, 1991; Shephard *et al.*, 2004).

The role of myofibroblasts in wound healing has been studied extensively and their crucial involvement has been verified both in experimental settings and in lesions encountered in diagnostic pathology. Myofibroblasts are present in several benign/reactive processes such as nodular fasciitis, liver cirrhosis, pulmonary fibrosis, and kidney fibrosis (Dayan *et al.*, 2005; Desmouliere *et al.*, 2005). Also in some malignant tumors, for example myofibrosarcomas, the malignant cells are best characterized as myofibroblasts (Lagace *et al.*, 1999; Montgomery *et al.*, 2001). The most common immunohistochemical markers used to detect myofibroblasts in clinical specimens are α-SMA and calponin (Tomasek *et al.*, 2002). Since myofibroblasts do not usually express desmin, it can be used to differentiate smooth muscle cells from myofibroblasts. We studied the expression of palladin both in an experimental wound model on rats and in human lesions known to contain high numbers of myofibroblasts (Dayan *et al.*, 2005). In

experimental wounds palladin 4Ig was neo-expressed in the granulation tissue fibroblasts and its expression preceded α-SMA. Palladin 4Ig was already present in proto-myofibroblasts, which contain actin stress fibers but are α-SMA negative. This *in vivo* result is in accordance with the results obtained *in vitro* with TGF-β1-treated cultured human dermal fibroblasts. Furthermore, a clear correlation between α-SMA and 4Ig expression was also seen in human myofibroblastic lesions such as scar tissue and nodular fasciitis.

Taken together we have shown that palladin expression is upregulated by TGF-β1 during myofibroblast differentiation. The palladin 4Ig isoform thus adds to the list of myofibroblast markers and probably participates in their force generation/transmission machinery.

MATERIALS AND METHODS

Palladin constructs

Palladin isoforms were named according to the number of Ig domains contained in the sequence (Figure 1). Palladin 3Ig corresponds to the previously characterized 90 kDa major isoform. The 3Ig constructs were PCR amplified using expressed sequence tag Ab023209 cDNA as a template. The 3Ig pEGFP has been described before (Rönty *et al.*, 2004). Unpublished human 4Ig 5′-sequence was reverse transcriptase-PCR amplified using U251 cell total RNA and cloned into pGEX4T-1 vector for recombinant protein production and into pEGFP-C/N vectors for mammalian cell expression. The identity of all derived constructs was verified by DNA sequencing.

Antibody production and Western blotting

Polyclonal antibodies were raised in rabbits using recombinant glutathione-*S*-transferase-fusion proteins containing N-terminal sequences of either the 3Ig or the 4Ig isoform as antigens. After four immunizations, rabbits were bled. The specificity of the antisera was tested by Western blotting of COS-7 cells transfected with cDNA of the 3Ig-full length sequence or 4Ig 5′-sequence in GFP vector. Transfection was verified by immunofluorescence microscopy. The palladin mAbs and the polyclonal C-terminal peptide antibody (953) have been described before (Parast and Otey, 2000; Mykkänen *et al.*, 2001). In addition, a polyclonal antibody raised against the N-terminal mouse 4Ig sequence (Ab-4Ig-Mo) was used (Rachlin and Otey, 2006). For Western blotting, U251 cells and human fibroblasts treated either with TGF-β1 (5 ng/ml) or vehicle were lysed in Laemmli buffer. Lysates were resolved in 10% SDS-PAGE and transferred onto nitrocellulose filters. The filters were probed with palladin antibodies, followed by peroxidase-conjugated swine anti-rabbit or anti-mouse IgG (DAKO, Glostrup, Denmark), and detected by enhanced chemiluminescence (Pierce, Rockford, IL). β-Actin was detected with a mAb (Clone AC-74, Sigma, St Louis, MO).

Cell cultures, transfections, and immunofluorescence microscopy

Human dermal and gingival fibroblasts (Leivonen *et al.*, 2005) and U251 cells were grown in modified eagles medium supplemented with 10% fetal calf serum, L-glutamine, and antibiotics. Fibroblasts were treated with recombinant TGF-β1 (Sigma) at the concentration of 5 ng/ml for 12–96 hours. Treated cells grown on glass coverslips were fixed in 3.5% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with palladin 3Ig or 4Ig antibodies together with α-SMA mAb (clone 1A4, Sigma). The antibodies were detected with FITC- and tetramethyl rhodamine isothiocyanate)-conjugated anti-rabbit and anti-mouse antibodies (Jackson Immunochemicals, West Grove, PA). The samples were analyzed in Zeiss Axiophot fluorescence microscope equipped with AxioCam cooled charge-coupled device camera.

Transduction of fibroblasts with recombinant adenoviruses

The adenoviral constructs RAdSmad2, RAdSmad3, and RAdSmad4 harboring human Smad2, Smad3, and Smad4 cDNAs, respectively, and an empty control virus, RAdpCA3, have been described (Leivonen *et al.*, 2002). Recombinant adenoviruses for Smad7 (RadSmad7) (Fujii *et al.*, 1999) and RAdSmad3DN (Pardali *et al.*, 2000) were kindly provided by Dr Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). Adenovirus for constitutively active MEK1 (RAdMEK1CA) (Foschi *et al.*, 1997) was kindly provided by Dr Marco Foschi (University of Florence) and for constitutively active MKK3b (RAdMKK3bE) (Wang *et al.*, 1998b) and for wild-type p38α with a FLAG tag (RAdp38α) (Wang *et al.*, 1998a) by Dr Jiahui Han (Scripps Research Institute, La Jolla, CA). Control adenovirus RAd66 (Wilkinson and Akrigg, 1992) was kindly provided by Dr Gavin W.G. Wilkinson (University of Cardiff, Cardiff, UK). Adenoviral infections of human gingival fibroblasts at multiplicity of infection 500 were performed as described (Leivonen *et al.*, 2002). p38 MAPK inhibitor SB203580 (10 μM) and MEK1/2 inhibitor PD98059 (30 μM) were from Calbiochem (San Diego, CA).

Northern blot hybridization

Total cellular RNA was extracted with Qiagen's Rapid RNA Purification kit (Qiagen, Chatsworth, CA) and northern blot hybridization was performed as described (Leivonen *et al.*, 2002). For the hybridization a 1.0-kb 5′ 4Ig palladin cDNA and a 1.3-kb rat glyceraldehyde-3-phosphate dehydrogenase cDNA were used (Fort *et al.*, 1985).

Animal experiments, immunofluorescence, and confocal microscopy

A total of 12 female Wistar rats (200–220 g) were used. After shaving the skin, full thickness $25 \times 25\,mm^2$ wounds, including the cutaneous muscle, were made using surgical scissors in the middle of the dorsum on the 1st day of the experiments and were allowed to heal spontaneously. Rats were killed by CO_2 anesthesia and granulation tissue was harvested after 3, 6, and 9 days post-wounding. Cryostat sections of 3 μm thickness were produced and immunostained. As primary antibody we used anti-α-SMA mAb (IgG1, a gift of Dr C. Chaponnier, University of Geneva) (Skalli *et al.*, 1986) and as secondary antibody FITC-conjugated goat anti-mouse IgG1 (Southern Biotechnology Associates Inc., Birmingham, AL). F-actin was probed with Alexa 647-conjugated phalloidin (Molecular Probes, Eugene, OR). Images were acquired with a confocal microscope (DM RXA2 with a laser scanning confocal head TCS SP2 AOBS, Leica, Glattbrugg, Switzerland), equipped with objective × 60/1.4 (Leica).

Immunohistochemistry

Human tissue samples obtained from operations performed at the Helsinki University Central Hospital, and archived at the Department of Pathology, University of Helsinki were used. Three to five

specimens per diagnosis were selected. Specimens were fixed in 10% buffered formal saline and embedded in paraffin. For routine histochemistry, 5 μm sections were stained with hematoxylin and eosin. Immunostaining was performed using the avidin–biotin peroxidase complex method (Dako Cytomation). For antigen retrieval, the slides were incubated in 10 mM sodium citrate buffer (pH 6.0) for 10 minutes at 95°C. Palladin was detected with two polyclonal antibodies (Ab-4Ig-Mo and Ab3Ig, both at dilution 1:500), α-SMA (clone 1A4 Sigma, dilution 1:10,000), and desmin (clone D33,Calbiochem, dilution 1:200) with mAbs.

The human and animal studies were approved by the authors' Institutional Review Boards. Tissue specimens were used under patient's consent and in agreement with the the Declaration of Helsinki Principles.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank H.M. Ahola, S. Kollanus, and J. Smith-Clerc for excellent technical assistance , Drs A. Moustakas, M. Foschi, J. Han, and G.W.G. Wilkinson for viral constructs, Dr C. Chaponnier for SMA antibody and Dr J.-J. Meister for providing laboratory facilities. The work was supported by the Sigrid Juselius Foundation, the Academy of Finland, the Finnish Heart Association, and the Helsinki and Turku University Hospital Research Funds. The work of B.H. was supported by the Swiss National Science Foundation, Grant #3100A0-102150/1. The work of C.O. was supported by NIH Grants GM61743 and NSY3253.

REFERENCES

Bang ML, Mudry RE, McElhinny AS, Trombitas K, Geach AJ, Yamasaki R *et al.* (2001) Myopalladin, a novel 145-kilodalton sarcomeric protein with multiple roles in Z-disc and I-band protein assemblies. *J Cell Biol* 153:413–27

Boukhelifa M, Hwang SJ, Valtschanoff JG, Meeker RB, Rustioni A, Otey CA (2003) A critical role for palladin in astrocyte morphology and response to injury. *Mol Cell Neurosci* 23:661–8

Boukhelifa M, Moza M, Johansson T, Rachlin A, Parast M, Huttelmaier S *et al.* (2006) The proline-rich protein palladin is a binding partner for profilin. *FEBS J* 273:26–33

Boukhelifa M, Parast MM, Bear JE, Gertler FB, Otey CA (2004) Palladin is a novel binding partner for Ena/VASP family members. *Cell Motil Cytoskeleton* 58:17–29

Chew CS, Chen X, Parente JA Jr, Tarrer S, Okamoto C, Qin HY (2002) Lasp-1 binds to non-muscle F-actin *in vitro* and is localized within multiple sites of dynamic actin assembly *in vivo*. *J Cell Sci* 115:4787–99

Darby I, Skalli O, Gabbiani G (1990) Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest* 63:21–9

Dayan D, Nasrallah V, Vered M (2005) Clinico-pathologic correlations of myofibroblastic tumors of the oral cavity: 1. nodular fasciitis. *J Oral Pathol Med* 34:426–35

Derynck R, Zhang YE (2003) Smad-dependent and smad-independent pathways in TGF-beta family signalling. *Nature* 425:577–84

Desmouliere A, Chaponnier C, Gabbiani G (2005) Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen* 13:7–12

Desmouliere A, Darby IA, Gabbiani G (2003) Normal and pathologic soft tissue remodeling: role of the myofibroblast, with special emphasis on liver and kidney fibrosis. *Lab Invest* 83:689–1707

Desmouliere A, Gabbiani G (1995) Myofibroblast differentiation during fibrosis. *Exp Nephrol* 3:134–9

Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G (1993) Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 122:103–11

Desmouliere A, Guyot C, Gabbiani G (2004) The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. *Int J Dev Biol* 48:509–17

Dugina V, Fontao L, Chaponnier C, Vasiliev J, Gabbiani G (2001) Focal adhesion features during myofibroblastic differentiation are controlled by intracellular and extracellular factors. *J Cell Sci* 114:3285–96

Fort P, Marty L, Piechaczyk M, Dani C, Jeanteur P, el Sabrouty S *et al.* (1985) Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucl Acids Res* 13:1431–42

Foschi M, Chari S, Dunn MJ, Sorokin A (1997) Biphasic activation of p21ras by endothelin-1 sequentially activates the ERK cascade and phosphatidylinositol 3-kinase. *EMBO J* 16:6439–51

Fujii M, Takeda K, Imamura T, Aoki H, Sampath TK, Enomoto S *et al.* (1999) Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. *Mol Biol Cell* 10:3801–13

Gabbiani G (2003) The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 200:500–3

Hashimoto S, Gon Y, Takeshita I, Maruoka S, Horie T (2001a) IL-4 and IL-13 induce myofibroblastic phenotype of human lung fibroblasts through c-jun NH2-terminal kinase-dependent pathway. *J Allergy Clin Immunol* 107:1001–8

Hashimoto S, Gon Y, Takeshita I, Matsumoto K, Maruoka S, Horie T (2001b) Transforming growth factor-beta1 induces phenotypic modulation of human lung fibroblasts to myofibroblast through a c-Jun-NH2-terminal kinase-dependent pathway. *Am J Respir Crit Care Med* 163:152–7

Hinz B, Celetta G, Tomasek JJ, Gabbiani G, Chaponnier C (2001) Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell* 12:2730–41

Hinz B, Dugina V, Ballestrem C, Wehrle-Haller B, Chaponnier C (2003) Alpha-smooth muscle actin is crucial for focal adhesion maturation in myofibroblasts. *Mol Biol Cell* 14:2508–19

Hinz B, Gabbiani G (2003a) Cell–matrix and cell–cell contacts of myofibroblasts: role in connective tissue remodeling. *Thromb Haemost* 90:993–1002

Hinz B, Gabbiani G (2003b) Mechanisms of force generation and transmission by myofibroblasts. *Curr Opin Biotechnol* 14:538–46

Hu B, Wu Z, Phan SH (2003) Smad3 mediates transforming growth factor-beta-induced alpha-smooth muscle actin expression. *Am J Respir Cell Mol Biol* 29:397–404

Lagace R, Seemayer TA, Gabbiani G, Schurch W (1999) Myofibroblastic sarcoma. *Am J Surg Pathol* 23:1432–5

Leivonen SK, Chantry A, Häkkinon L, Han J, Käthäri VM (2002) Smad3 mediates transforming growth factor-beta-induced collagenase-3 (matrix metalloproteinase-13) expression in human gingival fibroblasts. Evidence for cross-talk between Smad3 and p38 signaling pathways. *J Biol Chem* 277:46338–46

Leivonen SK, Häkkinen L, Liu D, Käthäri VM (2005) Smad3 and extracellular signal-regulated kinase 1/2 coordinately mediate transforming growth factor-beta-induced expression of connective tissue growth factor in human fibroblasts. *J Invest Dermatol* 124:1162–9

Luo H, Liu X, Wang F, Huang Q, Shen S, Wang L *et al.* (2005) Disruption of palladin results in neural tube closure defects in mice. *Mol Cell Neurosci* 29:507–15

Malmstrom J, Lindberg H, Lindberg C, Bratt C, Wieslander E, Delander EL *et al.* (2004) Transforming growth factor-beta 1 specifically induce proteins involved in the myofibroblast contractile apparatus. *Mol Cell Proteomics* 3:466–77

Massague J (2000) How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 1:169–78

Massague J, Wotton D (2000) Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J* 19:1745–54

Mattey DL, Dawes PT, Nixon NB, Slater H (1997) Transforming growth factor beta 1 and interleukin 4 induced alpha smooth muscle actin expression

and myofibroblast-like differentiation in human synovial fibroblasts *in vitro*: modulation by basic fibroblast growth factor. *Ann Rheum Dis* 56:426–31

Montgomery E, Goldblum JR, Fisher C (2001) Myofibrosarcoma: a clinicopathologic study. *Am J Surg Pathol* 25:219–28

Mykkänen OM, Grönholm M, Rönty M, Lalowski M, Salmikangas P, Suila H *et al.* (2001) Characterization of human palladin, a microfilament-associated protein. *Mol Biol Cell* 12:3060–73

O'Kane S, Ferguson MW (1997) Transforming growth factor beta s and wound healing. *Int J Biochem Cell Biol* 29:63–78

Parast MM, Otey CA (2000) Characterization of palladin, a novel protein localized to stress fibers and cell adhesions. *J Cell Biol* 150:643–56

Pardali K, Kurisaki A, Moren A, ten Dijke P, Kardassis D, Moustakas A (2000) Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. *J Biol Chem* 275:29244–56

Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB (1999) Myofibroblasts. I. paracrine cells important in health and disease. *Am J Physiol* 277:C1–9

Rachlin AS, Otey CA (2006) Identification of palladin isoforms and characterization of an isoform-specific interaction between Lasp-1 and paladin. *J Cell Sci* 119:995–1004

Ravanti L, Hakkinen L, Larjava H, Saarialho-Kere U, Foschi M, Han J *et al.* (1999a) Transforming growth factor-beta induces collagenase-3 expression by human gingival fibroblasts via p38 mitogen-activated protein kinase. *J Biol Chem* 274:37292–300

Ravanti L, Heino J, Lopez-Otin C, Kahari VM (1999b) Induction of collagenase-3 (MMP-13) expression in human skin fibroblasts by three-dimensional collagen is mediated by p38 mitogen-activated protein kinase. *J Biol Chem* 274:2446–55

Rönty M, Taivainen A, Moza M, Kruh GD, Ehler E, Carpen O (2005) Involvement of palladin and alpha-actinin in targeting of the Abl/Arg kinase adaptor ArgBP2 to the actin cytoskeleton. *Exp Cell Res* 310:88–98

Rönty M, Taivainen A, Moza M, Otey CA, Carpen O (2004) Molecular analysis of the interaction between palladin and alpha-actinin. *FEBS Lett* 566:30–4

Rubbia-Brandt L, Sappino AP, Gabbiani G (1991) Locally applied GM-CSF induces the accumulation of alpha-smooth muscle actin containing myofibroblasts. *Virchows Arch Cell Pathol Incl Mol Pathol* 60:73–82

Salmikangas P, Mykkänen OM, Grönholm M, Heiska L, Kere J, Carpen O (1999) Myotilin, a novel sarcomeric protein with two Ig-like domains, is encoded by a candidate gene for limb-girdle muscular dystrophy. *Hum Mol Genet* 8:1329–36

Shephard P, Hinz B, Smola-Hess S, Meister JJ, Krieg T, Smola H (2004) Dissecting the roles of endothelin, TGF-beta and GM-CSF on myofibroblast differentiation by keratinocytes. *Thromb Haemost* 92:262–74

Skalli O, Ropraz P, Trzeciak A, Benzonana G, Gillessen D, Gabbiani G (1986) A monoclonal antibody against alpha-smooth muscle actin: a new probe for smooth muscle differentiation. *J Cell Biol* 103:2787–96

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA (2002) Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 3:349–63

Tomasetto C, Moog-Lutz C, Regnier CH, Schreiber V, Basset P, Rio MC (1995) Lasp-1 (MLN 50) defines a new LIM protein subfamily characterized by the association of LIM and SH3 domains. *FEBS Lett* 373:245–9

Wang Y, Huang S, Sah VP, Ross J Jr, Brown JH, Han J *et al.* (1998a) Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J Biol Chem* 273:2161–8

Wang Y, Su B, Sah VP, Brown JH, Han J, Chien KR (1998b) Cardiac hypertrophy induced by mitogen-activated protein kinase kinase 7, a specific activator for c-Jun NH2-terminal kinase in ventricular muscle cells. *J Biol Chem* 273:5423–6

Wilkinson GW, Akrigg A (1992) Constitutive and enhanced expression from the CMV major IE promoter in a defective adenovirus vector. *Nucl Acids Res* 20:2233–9