るため，FDAへの申請をする必要がないのであろう。こうした2重構造が米国では問題となっており，FDAではコンパニオン診断薬を含めた新しい診断法に対するコントロールを強めたいという動きがある[14]。一方で，こうしたLDT的な診断を日本でも臨床応用するためには，米国と日本での健康保険システムの差が障壁になり，現行の制度の下では普及は難しいと考えられる。

2.3 タンパク質バイオマーカー

タンパク質バイオマーカーの検出には一般に抗体が用いられ，癌の診断においては病理組織標本上での免疫組織化学染色が主流である。乳癌におけるHER2，非小細胞肺癌等におけるEGFR過剰発現の検出がこれにあたる（表1）。抗体を用いる場合においては，当然抗体の特異性，反応性が診断のキーポイントとなり，いかに良い抗体を得るかが重要となるとともに，その評価にも注意を払わないといけない。分子標的薬の開発においては，既にターゲットが明らかとなっておりそれに対する抗体を得ることも容易であるため，初期より分子標的の診断とカップルして開発を進められることになるが，組織標本を得ることが難しいため，前立腺特異抗原（PSA）のように血中，もしくは尿など非侵襲的に得られる試料を使って検査ができるバイオマーカーがより望まれることになる。そうした観点からは，血液や尿を用いたプロテオーム解析によるバイオマーカー探索が有効である。

3. バイオマーカーのバリデーション

バイオマーカーのバリデーションは，その開発のされ方により異なる。分子標的薬に付随するバイオマーカーのように，既にターゲットが絞り込まれており，機能的にもバイオマーカーの役割に対する科学的根拠が確立されている場合には，主にバイオマーカーの測定方法のバリデーションが中心となる。薬物代謝酵素の多型のように，標的は絞り込まれ科学的な説明がある程度可能であり，その測定法に関しても確立されつつある事例もあるが，この場合，測定法の信頼性に加え，対象となる薬剤の感受性に関してその多型が実際に影響を与えるかどうかを，臨床データより適切に評価することが重要となる。また，遺伝子多型に関しては特に人種差の問題も大きく，海外でのデータをそのまま日本人集団に適応できないケースもある。未知の多型の影響や，遺伝子型以外のファクターの影響なども十分考慮する必要があり，注意が必要である。特に稀な遺伝子多型の場合には，十分な数の患者数が得られずバリデーションが難しい場合も想定されるが，このような場合には，臨床データ以外の科学的知見からそれを補完するとともに，バイオマーカーとしての利用後もプロスペクティブに症例を蓄積し事後評価を行うことが重要である。

次に，トランスクリプトームやプロテオーム，メタボロームといった網羅的解析からバイオ

マーカーを絞り込むというケースに関しては，測定法の妥当性に加え，得られたバイオマーカーが真にバイオマーカーとなりうるかをバリデーションすることが重要となる。得られたバイオマーカー分子が，目的とする疾患の発生や薬剤の作用に対して機能的に直接関与していることが科学的に証明されれば，この問題はある程度クリアーされるが，例えばマイクロアレイを用いた複数の遺伝子の発現情報から，あるアルゴリズムに基づいて診断を下すというような場合にはこの点が明確でなく，バイオマーカーとしての各遺伝子のバリデーションというよりも，多変量を用いて診断結果を導くためのアルゴリズムの妥当性の検証が重要となる。しかし，アルゴリズムの確立には様々な手法があり，標準的な手法は存在しないこともあり，アルゴリズムの導き方自体を評価することは大変難しい。そこで重要となるのは，十分な数と質の臨床性能試験のデータを使ってそのアルゴリズム即ち診断法の妥当性を検証することである。この点については，開発の段階から診断アルゴリズム（マーカー遺伝子の選択を含め）を導くためのデータセットとは別に，それとは完全に独立した新規のデータセットを用意して，バリデーションを行うことが，臨床性能試験を成功させる上でも重要となる[15]。

こうして開発したバイオマーカーが，体外診断薬として一般に普及する場合には，必ず薬事法に基づく承認申請を経る事になるので，すでにバリデーションが確立したバイオマーカーとして使用できる訳であるが，新規医薬品の開発過程における対象患者選択のためのバイオマーカーといった，いわゆる「コンパニオン診断薬」の場合には，十分なバリデーションを行った後に使用するという形態は取りづらく，現状では開発者の判断にゆだねられている。しかし，新規医薬品としての申請時には，用いた診断法のバリデーションに関しても評価されることになるため，あらかじめ準備が必要となる。最近では，新薬の申請においてファーマコジェノミクスに関するデータの利用，提出が国内外ともに推奨されているが，こうしたコンパニオン診断薬に対する評価基準は存在しない。今後，コンパニオン診断薬の利用を促進する意味でも，何等かの基準やルール作りが必要なのかも知れない。

いずれにしても，バイオマーカーのバリデーションに関しては，適切に計画された臨床性能試験の結果が最も重要であり，臨床性能試験自体に対してもガイドラインの整備が望まれている。

4. タンパク質バイオマーカーの診断への展開

これまで述べてきたように，ヒトゲノム解析と遺伝子解析技術の進歩により，遺伝子多型に基づくゲノムの個人差に関する理解が深まり，薬剤感受性の個人差を積極的に利用した個の医療の実現に向け，遺伝子診断の重要性が高まっている。一方で，プロテオーム解析技術の進歩もあり，再びバイオマーカーとしてのタンパク質ターゲットにも注目が集まっている。遺伝子上に情報は

あるが，遺伝子情報（配列）のみで全てが予測できる訳ではなく，リン酸化等の翻訳後修飾を含めた直接機能分子としてのタンパク質が，より有効なバイオマーカーとなり得るという点は議論の余地はない。この場合バイオマーカー探索においては，主に質量分析装置を用いたプロテオーム解析が用いられるが，バイオマーカーとして絞込みが行われた後は，通常の診断薬と同様，抗体を用いた酵素免疫化学的検出法が用いられることになる。分子標的薬のように，既にターゲットが決まっている場合には，それに対する抗体を作成するところから始まり，同様な課程を経て診断へと応用されていく。乳癌における HER2 や非小細胞肺癌における EGFR の発現等，分子標的薬のターゲットとして，その過剰発現が薬の効果を予測する上での診断として既に臨床応用されているケースがある。こうしたケースでは，メカニズム上治療効果の期待できない患者に対して無駄な投与を避けるという意味で，医療経済学的にも有益である。将来医療費の高騰という問題を抱えるわが国においては，医療費の削減という観点から，無駄な投薬を避けるということは大事であり，個別化医療の実現がそれに寄与することが期待される。今後，診断薬自体の認可に際しても，こうした医療経済学的な側面を積極的に評価しても良いだろう。

5. 新薬開発におけるタンパク質バイオマーカーの利用

新薬の開発段階においては，治療効果が期待できる患者のみを選択して治験を行うことにより，薬剤の有効性をより明確にすることができるというメリットがある。また，副作用を予測できるバイオマーカーを利用した場合には，従来は副作用が発生して開発を断念したようなケースにおいても，そうしたリスクを回避して市販化できる可能性がでてくる。また，逆にタンパク質バイオマーカーの探索から新薬の開発へつながるケースも出てくるであろう。特にこれまで有効な薬剤が存在しなかった疾患に関して，プロテオーム解析により新たなバイオマーカーが見つかれば，診断と合わせて新薬開発のターゲットともなり，診断が薬の開発とカップルして成り立つことになる。コンパニオン診断薬の理想的な形でもあり，開発におけるコンパニオン診断薬がそのまま市販後の診断のための体外診断薬として使用できるわけである。今後いっそうコンパニオン診断薬の利用は進むと考えられ，こうしたケースもでてくることが予想される。

6. 個の医療の実現に向けた規制の動向

コンパニオン診断薬は，少なくとも薬の開発過程で使用する場合には診断薬として認可を受けている必要性はなく，企業側の自己責任の上でその有効性に関しては担保されるべきものである。ただし，SNP 解析などある程度は一般性のある検査法に関しては，外部検査機関において

試験された結果を利用するケースも想定される。この場合，その方法が体外診断薬として認可されているものであれば問題ないが，それほど一般性が高くない場合が多いと予想されるためそのようなケースは稀であろう。米国における CLIA 認証のように，検査機関を評価して認証を与えるようなシステム作りが今後必要かもしれない。若しくは，LDT に近いコンパニオン診断薬としての新しいカテゴリーでの体外診断薬の承認システムが，それらの質と有効性を担保する上で必要となるかもしれない。

マイクロアレイを使った遺伝子型判定や発現解析という従来にはない手法を用いた診断には未知な部分も多いため，十分な有効性を担保するためにはどうしても慎重にならざるを得ない。しかし，慎重になればなるほど，承認申請にかかる手間と時間は大きくなり，有効な診断法が臨床応用されるまでの道のりが険しくなる。開発側も特にベンチャー企業のような場合には，途中で財政的に持たなくなるケースも多くなるであろう。

今後個別化医療の実現に向けて，個人の薬剤感受性等を予測するための体外診断薬および新薬開発におけるコンパニオン診断薬の利用を普及させるためには，これらの有効性，信頼性を的確に評価するためのガイドラインの整備が，規制する側，開発する側にとっても重要である。こうした考えの元，厚生労働省と経済産業省が協力して，次世代医療機器ガイドライン策定事業の中でテーラーメード医療用診断機器として DNA チップ等を用いた新しい診断法に関する評価指標の策定が進められている。既に遺伝子型を判定するための DNA チップに関する評価指標は「DNA チップを用いた遺伝子型判定装置に関する評価指標」として平成 20 年 4 月 4 日に医薬食品局，審査管理課，医療機器審査管理室長通知（薬食機発第 0404002 号）として公表された[16]。現在は，遺伝子発現解析に関する DNA チップ等の診断機器に関する同様の評価指標作りが進められている。

医薬品市場のグローバリゼーションにあわせ，今後こうしたガイドラインの整備に関しては，国内だけではなく国際的な整合性をとっていく努力が必要である。

7. バイオマーカー実用化へ向けた課題

7.1 リスクマネージメント

どんな診断法も予測の正確性は 100％でないので，ある確率で誤診はありうる。重要なのは，その際のリスクマネージメントである。例えば遺伝子発現により癌の予後予測をする場合，予後良好と判断されれば，積極的な治療を行わない場合を想定する。この際に，実は予後が良かった患者を，予後不良と誤診して積極的に治療した場合，結果として再発せず予後良好であるので，これは治療の成果とも受けとめられ，誤診とは気がつかないであろうから，おそらく問題は生じ

ない。しかし，実際には本来不必要であった苦痛を伴う治療を受けたことで，患者には不利益となる。逆に，実は予後が悪く積極的な治療が必要であった患者が，予後良好と診断され治療を受けなかったが，再発したというケースは問題となる。この場合は，診断結果が間違ったため必要な治療を受けられなかったと理解される訳で，患者の側からしてみれば，告訴も辞さないような致命的な間違いと受けとめられかねない。即ち，予後予測により治療の有無を選択する場合，予後が良い側に間違えた場合にリスクが高い。これは通常の病気の診断でもしかりであり，常にある確率での誤診は存在しうるので，それを最小限に抑えるとともに，よりリスクの低い側へマネージメントすることは重要である。しかし，完全にゼロリスクにはできないので，ある程度の間違いを許容できるよう，患者へのリスクコミュニケーションも必要となる。副作用の全くない薬が存在しないように，完璧な診断薬も存在しないしないということを十分に理解してもらった上で使用することが，有効な診断法を積極利用し個別化医療を実現していく上で重要な前提となる。

承認申請においても，こうしたリスクを極力避けようとすれば，新しい診断法の実用化への道のりは険しくなる。誤診のリスクをきちんと認識してそれを許容しつつ，そこから派生するリスクを最小限に抑える努力が必要である。そして，それでも防ぎきれなかった患者の被害に対しては，必要に応じて公的な補償を行うことを考えておかないといけないであろう。

7.2　産業育成

個別化医療の実現に向けて，バイオマーカーに関する関心が高まり，診断薬分野における新たな市場が形成されつつあるが，DNA チップを用いた発現解析をはじめとして，研究，開発レベルにある製品は多いものの，実際に診断薬として市販化されているものは少ない。また，診断薬の市場に関しては，医薬品ほど規模が大きくなく，開発する側にとってもリスクを抱えながらなかなか大きな利益を得るところまでは到達できない。しかし，個の医療の実現に向けて，診断薬の重要性はますます高まると予想され，患者側のメリットも大きいと期待できる。また，効かない人への投薬を回避するという意味においては医療経済学的にも有益であり，医療費の削減にもつながる。こうしたベネフィットを見据えて，現状では市販化のハードルを乗り越えるために苦労している診断薬，医療機器産業を何らかの形でバックアックするシステムが，国家戦略として国際的な競争力を増す意味でも重要であると考える。従来の承認申請システムとは違った，新たな枠組み，スキームにより新しい診断薬を世に出すという道筋も必要であろう。

7.3　コストダウン

個の医療の実現に向けて産業育成が必要である反面，DNA チップのような一般には高価な診

断法が患者や医療費に対し負担を強いることも懸念される。この点に関しては，今後より安価な新しい測定技術の開発や診断の効率化，開発コストの削減等を通じて，より良い診断法が開発されることを期待したい。コンパニオン診断薬の場合には，診断薬のコストが薬価に反映されることとなるが，対象患者を選択できることのメリットにより，トータルの開発費用を抑えることができれば，結果としてコストダウンにつながる。個別化医療の実現に向けて，医療費の削減も念頭において開発が進むことを期待する。

7.4 生命倫理

遺伝子診断では，当然個人の遺伝子情報を調べることになることから，個人情報の保護，生命倫理に十分な注意を払う必要がある。タンパク質バイオマーカーの場合も，変異の検出であれば二次的に遺伝子の情報を解析することになるため，同様の注意が必要である。遺伝子情報を取り扱う上で，生殖細胞を介した遺伝的（先天的）な変化と，癌のように体細胞における後天的な変化は明確に区別される。前者は特に取り扱いに注意を払わなければいけないが，後者に関してはそれほど注意は必要ない。前者として難しいのは，SNP など比較的頻度の高い事例と，疾患等との関連性が明らかでない例に対してどれだけ慎重であるべきかという点であるが，いずれにしても，知り得た遺伝子情報により被験者が何らかの不利益を被らないよう，個人情報の保護には常に注意を払わなければならない。

おわりに

今我が国が直面する長寿高齢化社会における医療費の高騰という問題への対策として考えた場合，コンパニオン診断薬等の新しい技術は比較的高価な技術となるためそれ自身は医療費削減に逆行するものではあるが，結果として，より有効な薬を世に出すことができ，副作用を回避し，薬が効かない人への無駄な投与を減らせれば，医療経済的にも結果的には大きな利益をもたらすと期待できる。

DNA チップや次世代シークエンサーでは日本の技術は遅れを取ったが，今後市場が拡大すると予想されるコンパニオン診断薬を含めた新規の診断法に対する技術開発と臨床応用に関して巻き返しを図るためにも，国家的な戦略，ビジョンの確立が急務である。

文　　献

1) Redon R, et al., Global variation in copy number in the human genome. Nature 444, 444-454 (2006)

2）油谷浩幸，次世代シーケンサーによるがんゲノム解析，癌と化学療法 38，1-6（2011）
3）稲澤譲治，蒔田芳男，羽田 明編著，アレイ CGH 診断活用ガイドブック－知っておきたい染色体微細構造異常症－ 医薬ジャーナル社（2008）
4）http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm071455.pdf
5）Kricka LJ, Imai K, Fortina P, Analytical ancestry: evolution of the array in analysis. Clin Chem. 56, 1797-803. (2010)
6）戸田年総，荒木令江 編著，疾患プロテオミクスの最前線－プロテオミクスで病気を治せるか－ メディカルドゥ（2005）
7）上家潤一，Selected Reaction Monitoring（SRM）を用いた定量フォーカストプロテオミクス， 創薬・タンパク質研究のためのプロテオミクス解析（実験医学別冊 26）小田 吉哉 長野 光司編，羊土社，68-74（2010）
8）牛島俊和，塩田邦郎，田嶋正二，吉田 稔 編著，エピジェネティクスと疾患－基礎メカニズムと解析手法の最新知見からがん・神経・代謝・アレルギー疾患などへの応用まで（実験医学増刊 Vol. 28-15）羊土社（2010）
9）田口 良 編著，メタボロミクス：その解析技術と臨床・創薬応用研究の最前線（遺伝子医学 MOOK 16 号）メディカルドゥ（2010）
10）佐治英郎，田畑泰彦 編著，ますます広がる分子イメージング技術－生物医学研究から創薬，先端医療までを支える分子イメージング技術・DDS との技術融合－（遺伝子医学 MOOK 9 号）メディカルドゥ（2010）
11）http://www.info.pmda.go.jp/downfiles/ivd/PDF/700025_22100AMX00779000_A_01_02.pdf
12）http://www.sekisuimedical.jp/business/diagnostics/others/invader/index.html
13）上野貴之，戸井雅和，個別化医療 OncotypeDX® DNA チップ／マイクロアレイ臨床応用の実際－基礎，最新技術，臨床・創薬研究応用への実際から今後の展開・問題点まで－（遺伝子医学 MOOK 9 号）油谷浩幸編，メディカルドゥ（2010）
14）http://www.fda.gov/MedicalDevices/NewsEvents/WorkshopsConferences/ucm212830.htm
15）Subramanian J, Simon R, What should physicians look for in evaluating prognostic gene-expression signatures?, Nat Rev Clin Oncol. 7. 327-34. (2010)
16）http://dmd.nihs.go.jp/jisedai/dna/index.html

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Degradation of Filamin Induces Contraction of Vascular Smooth Muscle Cells in Type-I Collagen Matrix Honeycombs

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Key Words

Smooth muscle cell • Three-dimensional culture • Filamin • Cytoskeleton

Abstract

Background: Dedifferentiated rabbit vascular smooth muscle cells (SMCs) exhibit similar features to differentiated SMCs when cultured in three-dimensional matrices of type-I collagen called "honeycombs," but the mechanism is unknown. The role of filamin, an actin-binding protein that links actin filaments in SMCs, was investigated. Methods: Filamin and other related proteins were detected by western blot analysis and immunofluorescence staining. Honeycomb size was measured to confirm the contraction of SMCs. Results: Full-length filamin was expressed in subconfluent SMCs cultured on plates; however, degradation of filamin, which might be regulated by calpain, was observed in confluent SMCs cultured on plates and in honeycombs. While filamin was co-localized with β-actin in subconfluent SMCs grown on plates, filamin was detected in the cytoplasm in SMCs cultured in honeycombs, and degraded filamin was mainly detected in the cytoplasmic fraction of these cells. In addition, β-actin expression was low in the cytoskeletal fraction of SMCs cultured in honeycombs compared with cells cultured on plates, and the size of the honeycombs used for culturing SMCs was significantly reduced. Conclusion: These data suggest that degradation of filamin in SMCs cultured in honeycombs induces structural weakness of β-non-muscle actin filaments, thereby permitting SMCs in honeycombs to achieve contractility.

Introduction

Vascular smooth muscle cells (SMCs) undergo phenotypic changes upon differentiation and dedifferentiation. A shift from a dedifferentiated to a differentiated phenotype is a crucial event in the regression of atherosclerosis [1, 2]. In normal aortas, SMCs can contract when they differentiate, but in atherosclerotic lesions, SMCs undergo proliferation and lose the ability to contract after dedifferentiation [3]. SMCs in the normal arterial media are surrounded by components of the extracellular matrix (ECM), which is thought to maintain SMCs in the differentiated phenotype.

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If SMCs are isolated from the ECM and cultured in medium containing 5% serum, they maintain the dedifferentiated phenotype [4].

Dedifferentiated rabbit SMCs displayed unique features when cultured in three-dimensional matrices of type-I collagen called "honeycombs" [5]. They stopped proliferating despite the presence of sufficient serum in the medium, showed morphological changes and expressed caldesmon heavy chain (a marker of mature differentiated SMCs), all of which are features that are similar to those observed in aortic SMCs *in vivo*. SMCs in honeycombs are attached across the lumens of the honeycombs by a few adhesion points and have a more spindly shape than do SMCs cultured on plastic plates. We have also reported that formation of cellular cross-bridges in honeycombs is important for changes that occur in SMCs grown in honeycombs [6]. These results suggest that relationships exist between the structural rearrangement of the cytoskeleton and specific features of SMCs cultured in honeycombs.

It has been demonstrated that SMCs derived from freshly isolated gizzard smooth muscle strips possess a structural lattice that is composed of a contractile apparatus and a cytoskeleton (β-non-muscle actin filaments, intermediate filaments and dense bodies) [7, 8]. The main components of the contractile apparatus are known to be α-smooth muscle actin (α-actin), myosin, tropomyosin, calponin, caldesmon and myosin light chain kinase, while the cytoskeleton is mainly constructed of β-non-muscle actin (β-actin) filaments, filamin, calponin and vimentin. Dense bodies are composed of β-actin, α-actinin and calponin [9]. Of these proteins, α-actinin, an actin-binding protein localized to dense bodies and adherens junctions that is involved in bundling of actin filaments [8, 10, 11], and filamin, an actin cross-linking protein, are thought to affect the arrangement of actin filaments.

Three filamin genes (FLNA, FLNB and FLNC) have been identified in humans [12]. Filamin A has a molecular weight of 280 kDa and a molecular length of approximately 160 nm [13]. Filamin A consists of an N-terminal actin-binding domain (ABD) and a rod-like domain of 24 repeats, which is interrupted by 30-amino-acid long flexible loops, H1 (between repeats 15 and 16) and H2 (between repeats 23 and 24). The last repeat, repeat 24, represents the self-association domain of the molecule. Filamin dimers link actin filaments into orthogonal networks or parallel bundles, and an *in vitro* study has shown that the manner in which actin filaments are organized depends on the ratio of filamin to actin [14]. It has also been reported that filamin co-localizes with the non-muscle actin cytoskeleton and intermediate filaments, but not with the contractile apparatus, in SMCs derived from freshly isolated gizzard smooth muscle strips [7, 8]; these findings indicate that filamin mainly interacts with the β-non-muscle actin filaments in SMCs.

In the present study, we hypothesized that filamin would be expected to influence the arrangement of the cytoskeletal structure in SMCs. Filamin is known to be degraded at H1 and H2 sites by Ca^{2+}-dependent proteases known as calpains [15], including μ-calpain and m-calpain, which are ubiquitously expressed [16, 17]. We therefore investigated whether filamin in SMCs was degraded by calpain and whether SMCs cultured in honeycombs could contract. We also discuss the relationship between filamin and phenotypic changes in SMCs.

Materials and Methods

Honeycomb collagen tubes

Collagen sponges called honeycombs that consisted of type-I collagen were obtained from Koken Co. Ltd. (Japan). The structure of the honeycomb was porous and consisted of many tubes aligned side-by-side, similar to a beehive [5]. The pore diameters of the tubes were 200–300 μm. The honeycombs were cut vertically into cubes of dimensions 4 × 4 × 2 mm or 6 × 6 ×2 mm.

Cell culture

Animal experiments were carried out according to the "Principles of Laboratory Animal Care" (NIH publication number 85-23, revised 1985) and the Guidelines of the Animal Investigation Committee, Chiba University.

SMCs from the medial layer of the thoracic aorta of male Japanese white rabbits weighing ~2 kg were prepared using the explant method. Nembutal (Dainippon Sumitomo Pharma, Japan) was administered (25 mg kg^{-1}, i.v.) before the artery was extirpated. SMCs were cultured as described by Ishii et al. [5]. SMCs were treated with trypsin–EDTA (Sigma–Aldrich) for 1–2 minutes, and released SMCs were subcultured at 1 × 10^6 cells per dish (diameter, 10 cm) in 8 ml of 10% fetal bovine serum/Dulbecco's modified Eagle's medium (10% FBS-DMEM). Biochemical analyses (expression of α-actin and doubling time in proliferation) were conducted on SMCs prior to use. SMCs were subcultured every 2–4 days, and the process was repeated when cells reached confluence. SMCs used in this study were passages 4–7.

NIH3T3 cells were cultured in 10% FBS-DMEM under standard cell culture conditions (37 °C, 5% CO_2). Human umbilical vein endothelial cells (HUVEC, Lonza) were cultured under standard cell culture conditions (37 °C, 5% CO_2) in endothelial basal medium 2 (EBM2, Lonza) supplemented with human recombinant fibroblast growth factor-B (hFGF-B), human recombinant vascular endothelial growth factor (VEGF), human

recombinant epidermal growth factor (hEGF), hydrocortisone, heparin, ascorbic acid, R3-insulin-like growth factor-1, gentamicin/amphotericin B (GA) and 2% FBS, as suggested by the supplier.

The following procedure was used to prepare cells in honeycombs. First, cells cultured on plates were released by incubation with trypsin–EDTA and collected by centrifugation at 200 × *g* for 10 minutes at 25°C. Next, cells were suspended in 300–400 µl of culture medium and incubated with honeycombs (4 × 4 x 2 or 6 × 6 × 2 mm, 2 × 10^6 cells per 30 honeycombs) on a dish (diameter, 6 cm) for 3 hours at 37°C. Then, 3–4 ml of culture medium was added. The medium was changed every 2–3 days. ALLN was obtained from Calbiochem and added to 10% FBS-DMEM for experiments.

Cell number measurements

Cells were washed twice with PBS and released from plates by incubation with trypsin–EDTA at 37 °C for 1 minute or from honeycombs by incubation with collagenase-I (Sigma–Aldrich) at 37 °C for 30 minutes. After the cells were collected, cell number was determined using an improved Neubauer deep chamber hemocytometer. Cell viability was assessed by trypan blue exclusion.

Determination of honeycomb area

Honeycombs (6 × 6 × 2 mm) were incubated with or without cells for 0–7 days in culture medium. At days 0, 1, 3 and 7, pictures were taken of the honeycombs, with or without cells, immersed in medium in dishes (diameter, 6 cm) using a digital camera (Canon). The photographs were enlarged, and the areas (length x width) of the honeycombs were calculated using Image J 1.41.

Subcellular fractionation

Triton X-100-soluble (cytoplasmic) and -insoluble (cytoskeletal) fractions were prepared as previously described [18].

Western blotting

Detection of filamin, µ-calpain, m-calpain, phosphofilamin A (Ser^{2152}), calpastatin, vimentin, myosin heavy chain (MHC), tropomyosin, α-actinin, α-actin or β-actin was carried out by western blotting analysis.

SMCs were washed twice with PBS and released from plates by incubation with trypsin–EDTA at 37°C for 1 minute or from honeycombs by incubation with collagenase-I at 37°C for 30 minutes. SMCs were collected and washed twice with cold PBS. Cells were lysed in IP buffer [20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.5% sodium cholate, 0.5% Triton X-100, 5 mM NaF, 0.5 mM Na_3VO_4, and 1% protease inhibitor cocktail (Sigma–Aldrich)] for 20 minutes on ice. Cell lysates were centrifuged at 12,000 x *g* for 20 minutes, and protein concentrations were determined using a bicinchoninic acid protein assay reagent kit (Pierce). Bovine serum albumin was used as a standard in this assay. After lysates were separated on 6%, 8%, or 12% SDS-PAGE gels, proteins were transferred to an Immobilon membrane (Millipore) and immunoblotted with specific antibodies. Immunoblots were visualized using electrochemiluminescence (ECL) western blotting detection reagents (GE Healthcare) or Immobilon™ Western (Millipore). The primary antibodies used for western blotting were monoclonal anti-filamin antibody (1.BB.804; Abcam), polyclonal anti-calpain 1 (µ-calpain) antibody (H-65; Santa Cruz Biotechnology), polyclonal anti-calpain II (m-calpain) antibody (Chemicon International), polyclonal anti-α-actinin antibody (H-300; Santa Cruz Biotechnology), polyclonal anti-β-actin antibody (l-19; Santa Cruz Biotechnology), polyclonal anti-phosphor-filamin A (Ser^{2152}) (Cell Signaling), monoclonal anti-calpastatin antibody (PI-11; Santa Cruz Biotechnology), monoclonal anti-vimentin antibody (V9; Sigma–Aldrich), monoclonal anti-α-actin antibody (1A4; DAKO), monoclonal anti-myosin (smooth) antibody (HSM-V; Sigma–Aldrich) and monoclonal anti-tropomyosin antibody (TM311; Sigma–Aldrich). Membranes were incubated with antibody for 1–3 hours. Membranes were treated with anti-mouse or anti-rabbit IgG (horseradish-peroxidase-linked species-specific antibody; GE Healthcare) as the secondary antibody for 30 minutes.

Northern blotting

Total RNA was isolated from SMCs using Isogen reagent (Isogen International). Ten micrograms of total RNA was fractionated by electrophoresis in a 1% agarose gel containing 400 mM formaldehyde. RNA was transferred to a Hybond N^+ membrane (Amersham) by capillary transfer overnight and fixed to the membrane by UV crosslinking. Polymerase chain reaction (PCR)-derived probes for filamin A were prepared from the total cDNA of SMCs using a DIG DNA Labeling Kit (Roche). The primer pairs for probes were as follows: forward primer, 5'-GGG TCA CCT ACT GCC CCA C-3'; reverse primer, 5'-GGA TCT TGA GGC TGA GGT CG-3'. DIG Easy Hyb (Roche), DIG Wash and Block Buffer Set (Roche), DIG Luminescent Detection Kit (Roche) and CDP-Star ready-to-use (Roche) were used for hybridization. Detection was carried out according to the manufacturer's protocol.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from SMCs using Isogen reagent. cDNA was synthesized using 1 µg RNA in 20 µl of reaction mixture with Omniscript® Reverse Transcription (QIAGEN). One microliter of reverse-transcribed cDNA was used in a total reaction volume of 25 µl for PCR amplification using specific primers for filamin A. Amplification of glycerol-3-phosphate dehydrogenase (GAPDH) was used as a control. The following primer pairs were used: forward and reverse primers for filamin A were the same as those used for northern blotting; forward primer for GAPDH, 5'-G ATG CCC CCA TGT TTG TGA T-3'; reverse primer for GAPDH, 5'-AAG GCC ATG CCA GTG AGT TT-3'. Reaction products were analyzed by electrophoresis on 2% agarose gels and observed with ultraviolet transillumination after ethidium bromide staining. PCR cycling was as follows: initial denaturation at 94°C for 1 minute (one cycle), denaturation at 94°C for 10 seconds, annealing by Taq polymerase (Takara Bio Incorporated) at 69.3°C for filamin A or at 66.2°C for GAPDH for 10 seconds and elongation at 72°C for 30 seconds. The number of amplification cycles performed for filamin A and GAPDH were 27 and 22 respectively.

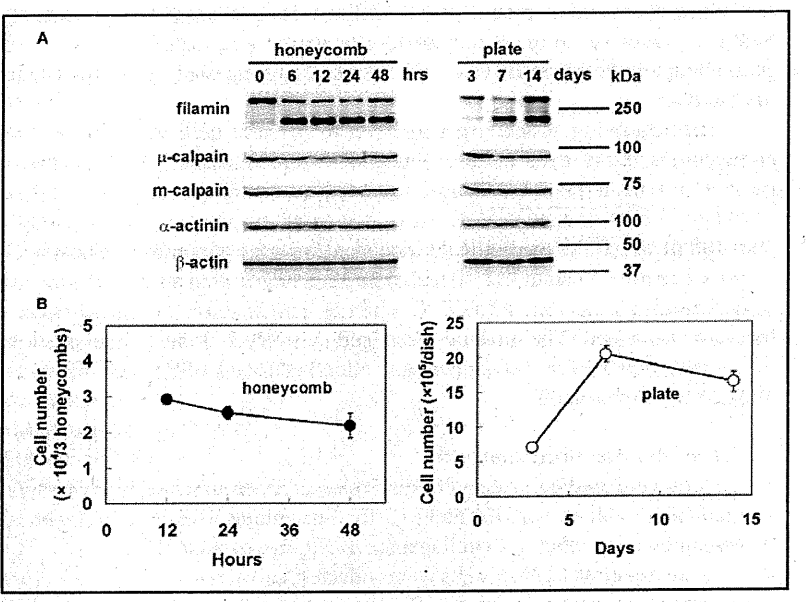

Fig. 1. Change in the molecular weight of filamin in SMCs cultured under different conditions. Honeycomb: SMCs cultured on a dish (diameter, 10 cm) were treated with trypsin-EDTA, and isolated cells (0 hour) were cultured in honeycombs for 6, 12, 24, and 48 hours (hrs in figures). Plate: SMCs were plated at 5 x 10^5 cells per dish (diameter, 10 cm) and cultured for 3, 7 and 14 days. (A) Western blot analysis of filamin and related proteins. (B) Cell growth of SMCs cultured in honeycombs and on plates. ●, SMCs cultured in honeycombs; ○, SMCs cultured on plates.

Immunofluorescence

SMCs on coverslips or in honeycombs were fixed in 3.7% formaldehyde in PBS for 10 minutes. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and pre-incubated with 10% normal goat serum (Wako) for 10 minutes to block non-specific binding. These cells were then incubated with monoclonal anti-filamin antibody (1.BB.804; Abcam) or monoclonal anti-α-actin antibody (1A4; DAKO) and polyclonal anti-β-actin antibody (1-19; Santa Cruz Biotechnology) for 3 hours. This was followed by incubation with FITC-conjugated anti-mouse IgG (Sigma–Aldrich) and TRITC-conjugated anti-rabbit IgG (Sigma–Aldrich) for 1 hour. Immunofluorescence was observed using a Fluoview FV500 (Olympus) laser-scanning microscope with a 60 x 1.00 NA water immersion objective. Images were digitally processed using Adobe Photoshop Elements 4.0 software (Adobe).

Statistical analysis

The statistical significance of differences was assessed by Tukey's test in Fig. 8B and D, and Dunnett's test in Fig. 8C. Data are mean ± S.E.

Results

Changes in the molecular weight of filamin in SMCs cultured under different conditions

Changes in the molecular weight of filamin in SMCs cultured under different conditions were investigated (Fig. 1). At 0 hours, the molecular weight of filamin was mainly 280 kDa. After SMCs were cultured for 6 hours in honeycombs, however, filamin with molecular weights of both 280 and 180 kDa were observed (Fig. 1A). Moreover, SMCs in honeycombs were spindle-shaped, adhered across the lumen of the honeycombs and stopped proliferating (Fig. 1B). The viability of these cells at 12, 24 and 48 hours was 85, 85 and 86%, respectively. These data are consisted with our previous study [5]. SMCs cultured on plates proliferated and became confluent on day 7 (Fig. 1B). The viability of the SMCs grown on plates on day 3, 7 and 14 was 88, 78 and 84%, respectively. Subconfluent SMCs cultured on plates mainly expressed filamin with a molecular weight of 280 kDa, but two types of filamin were apparent after these cells became confluent (Fig. 1A). Subconfluent SMCs were widely spread on plates, while confluent SMCs had a spindly shape. The aortic media of Japanese white rabbits expressed filamin of both 280 and 180 kDa (Fig. 2), and this pattern was identical to that of filamin in SMCs

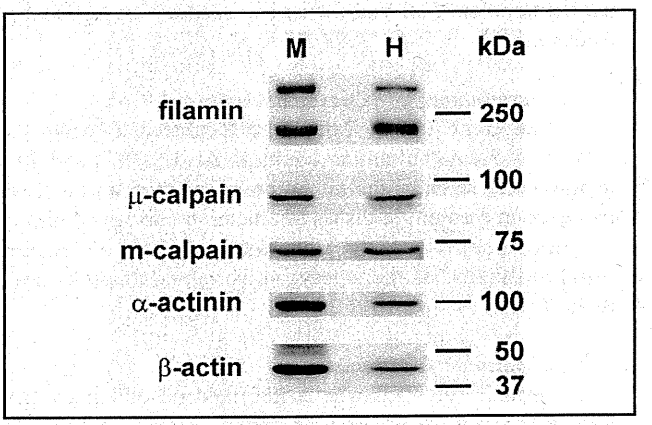

Fig. 2. Expression of filamin in aortic media. M, aortic media of Japanese white rabbits; H, SMCs cultured in honeycombs for 12 hours.

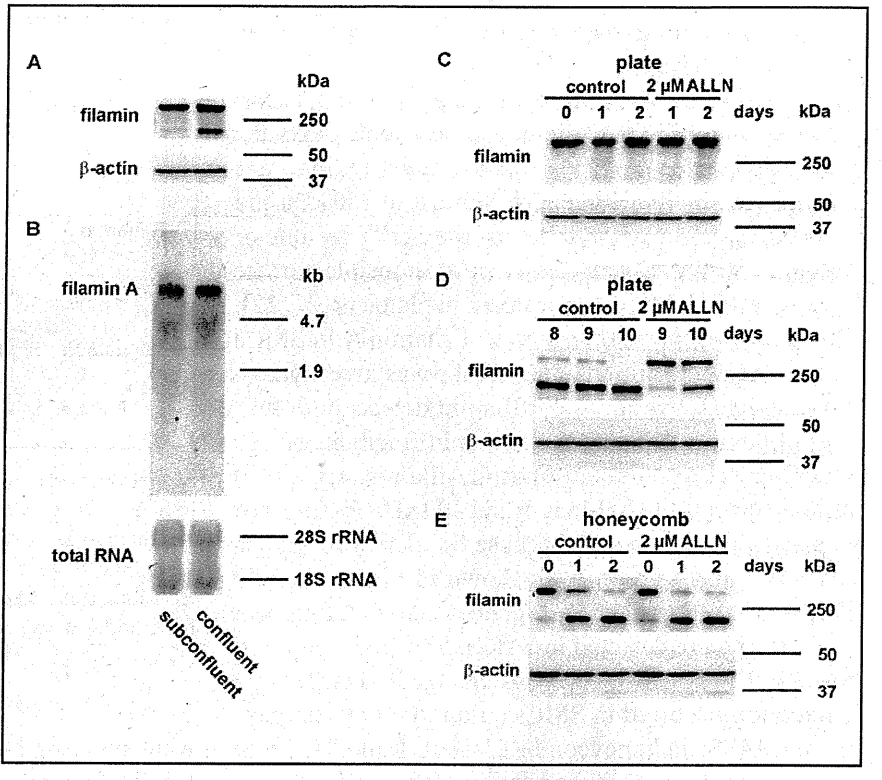

Fig. 3. Filamin degradation. (A) Western blot analysis of filamin in SMCs. SMCs were plated on a dish (diameter, 10 cm) and cultured until subconfluent or confluent. (B) Northern blot analysis of filamin A in SMCs cultured on plates. SMCs were plated on a dish (diameter, 10 cm) and cultured until subconfluent or confluent. Total RNA was isolated, northern-blotted and probed with filamin A. (C) Western blot analysis of filamin in SMCs. SMCs were plated at 2 x 10^5 cells per dish (diameter, 6 cm) and cultured for 24 hours. Next, SMCs were incubated with 2 μM ALLN (added at day 0) for 1 or 2 days. (D) Western blot analysis of filamin in SMCs. SMCs were plated at 2 x 10^5 cells per dish (diameter, 6 cm) and cultured for 8 days to reach confluence. Two micromolar ALLN was then added, and SMCs were incubated for 1 or 2 days. (E) Western blot analysis of filamin in SMCs. SMCs cultured on dishes (diameter, 10 cm) were incubated with 2 μM ALLN for 24 hours. SMCs were then treated with trypsin–EDTA, and isolated SMCs were cultured in honeycombs in the presence of 2 μM ALLN for 1 or 2 days.

cultured in honeycombs. The filamin antibody used in this experiment does not distinguish between filamin isoforms, but matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS) analysis using an AB4700 mass spectrometer (Applied Biosystems) confirmed that the 280 and 180 kDa forms of filamin in SMCs were the filamin A isoform (data not shown). μ-Calpain and m-calpain were expressed in SMCs under each of the culture conditions described above, and expression of α-actinin and β-actin was not altered by different culture conditions.

Degradation of filamin

To begin investigating how the molecular weight of filamin changed in SMCs, we initially examined whether this process involves regulation at the transcriptional level.

Filamin A mRNA was detected by northern blot analysis of total RNA isolated from subconfluent and confluent SMCs cultured on plates (Fig. 3A and B). Results from this study indicated that both the subconfluent and confluent SMCs expressed filamin A mRNA of only one size (~8.1 kb). Filamin transcripts in humans and mice are reported to be 8–9.5 kb and 9.5–10 kb, respectively [15, 19, 20]. Filamin A mRNA therefore appears to encode the entire filamin molecule; thus, it is likely that the change in the molecular weight of filamin observed in SMCs occurs after translation.

We next examined whether filamin was degraded by calpain in SMCs. Filamin expression was not altered upon incubated with 2 μM ALLN (N-Acetyl-Leu-Leu-Nle-CHO), an inhibitor of calpain, in subconfluent SMCs cultured on plates (Fig. 3C). In contrast, the change in the molecular weight of filamin was suppressed by 2 μM ALLN when confluent SMCs on plates at day 8 were incubated with 2 μM ALLN (Fig. 3D). These results indicate that filamin is degraded by calpain. When SMCs were cultured in honeycombs, filamin degradation was not suppressed by ALLN, even though SMCs cultured in honeycombs were incubated with 2 μM ALLN from day 1 (data not shown). The lack of an effect of ALLN on filamin degradation in this study might be due to the fact that ALLN was not incorporated into SMCs cultured in honeycombs. Next, SMCs were pre-incubated with 2 μM ALLN for 24 hours while still cultured on plates and then transferred to honeycombs with 2 μM ALLN; however, filamin degradation by calpain was still not suppressed by ALLN (Fig. 3E). When the concentration of ALLN was increased to 50 μM, most SMCs died, but living cells in honeycombs expressed filamin with molecular weights of both 280 and 180 kDa (data not shown).

Phosphorylation of filamin A and the presence of calpastatin

Next, we examined the regulation of filamin degradation in SMCs. Filamin is known to be degraded by calpain at residues 1761-1762 in the H1 region. As a result, filamin is divided into 2 fragments of 180 kDa and 100 kDa [15]. Phosphorylation of the Ser^{2152} residue of filamin A has been reported to be responsible for the degradation of filamin A by calpain in platelets [21, 22]. Thus, the phosphorylation status of filamin A in SMCs cultured on plates and in honeycombs was investigated. The monoclonal antibody for filamin detects both full-length filamin and a degraded filamin fragment of 180 kDa. Conversely, the anti-phospho-filamin A (Ser^{2152}) antibody detects the full-length and 100 kDa fragment of phosphorylated filamin A because the Ser^{2152} residue is in the 100 kDa fragment. As shown in Fig. 4, the 280 kDa form of filamin A and a degraded filamin A fragment of 100 kDa were detected in SMCs. Phosphorylation of the 280 kDa filamin A was not changed when filamin degradation occurred in SMCs cultured on plates (days 3, 6 and 14) or in honeycombs (days 0, 1 and 2). These results suggest that phosphorylation of the Ser^{2152} residue of filamin A is not responsible for the degradation of filamin A by calpain in SMCs under the conditions used in this study.

Calpastatin is known to be an endogenous inhibitor of calpain [23]; therefore, the relationship between presence of calpastatin and appearance of the 180 kDa filamin was investigated in SMCs. Calpastatin was present when 180 kDa filamin was absent in SMCs cultured on plates and in honeycombs (Fig. 4). Moreover, when the 180 kDa filamin appeared, calpastatin disappeared. Calpastatin mRNA levels were almost the same in subconfluent SMCs cultured on plates and those cultured in honeycombs, which did and did not express calpastatin protein, respectively (data not shown). These results suggest that filamin degradation in SMCs might be regulated by calpastatin expression.

Localization of filamin and β-non-muscle actin filaments in SMCs

Previous studies have shown that filamin mainly interacts with β-non-muscle actin filaments in SMCs [7, 8]. To clarify the intracellular distribution of filamin after degradation, we examined filamin and β-actin expression by immunofluorescence microscopy in SMCs. Filamin immunoreactivity was observed along fibers and co-localized with β-actin immunoreactivity in subconfluent SMCs cultured on plates (Fig. 5A and B). When SMCs

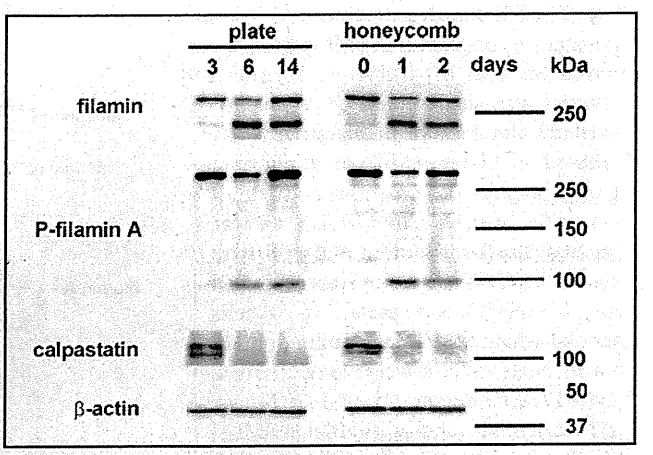

Fig. 4. Expression of phosphorylated filamin A and calpastatin in SMCs. Plate: SMCs were plated at 1.5 x 10^5 cells per well (6-well plate) and cultured for 3, 6 and 14 days. Honeycomb: SMCs cultured on a dish (diameter, 10 cm) were treated with trypsin–EDTA, and isolated cells (day 0) were cultured in honeycombs for 1 and 2 days.

were incubated with 0.5 μM cytochalasin D, this co-localization was disrupted, and both filamin and β-actin were widely dispersed in the cytoplasm (data not shown), suggesting that filamin interacts with β-non-muscle actin filaments. In honeycombs, filamin staining was evenly detected in the cytoplasm and was not stained as fibers in most cells (Fig. 5C and D). A small amount of filamin was co-localized with β-actin in a few cells (data not shown).

As shown in Fig. 6, only the 280 kDa form of filamin was detected in whole cell lysates of subconfluent SMCs cultured on plates, and it was mainly detected in the Triton X-100-insoluble (cytoskeletal) fraction. In honeycombs, both the 280 and 180 kDa forms of filamin were detected in whole cell lysates. Expression of the 180 kDa form of filamin in the Triton X-100-soluble (cytoplasmic) fraction was greater than that in the insoluble fraction, whereas the 280 kDa form of filamin was mainly detected in the insoluble fraction. Although β-actin expression was greater in the Triton X-100-insoluble fraction than in the soluble fraction in subconfluent SMCs grown on plates, expression of β-actin in SMCs cultured in honeycombs was almost the same in the Triton X-100-soluble and -insoluble fractions. μ-Calpain was detected only in the Triton X-100-soluble fraction. Vimentin, a major component of intermediate filaments, was mainly seen in the Triton X-100-insoluble fraction, as expected [24]. Based on the results presented in Figs. 5 and 6, it can be assumed that degraded filamin, which does not bind to β-non-muscle

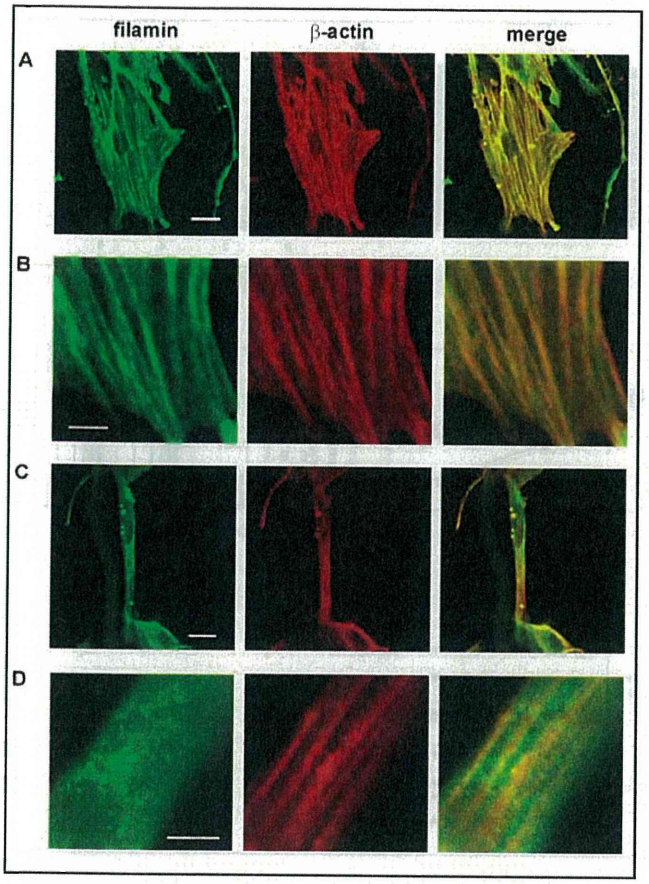

Fig. 5. Localization of filamin and β-actin in SMCs. (A, B) SMCs were cultured to subconfluence on a plate. (C, D) SMCs were cultured in a honeycomb for 3 days. Green indicates filamin, and red indicates β-actin. Scale bar, 20 μm (A, C); 5 μm (B, D).

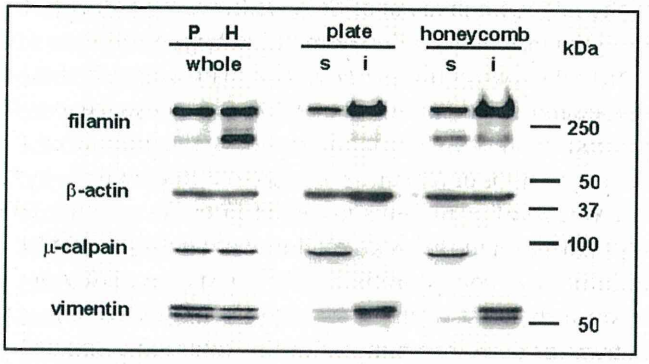

Fig. 6. Subcellular localization of filamin and β-actin between cytoplasmic and cytoskeletal fractions in SMCs. P, SMCs cultured to subconfluence on plates; H, SMCs cultured in honeycombs for 3 days; whole, whole cell lysates; s, Triton X-100-soluble fraction; i, Triton X-100-insoluble fraction.

actin filaments, is present in the cytoplasmic fraction in SMCs cultured in honeycombs.

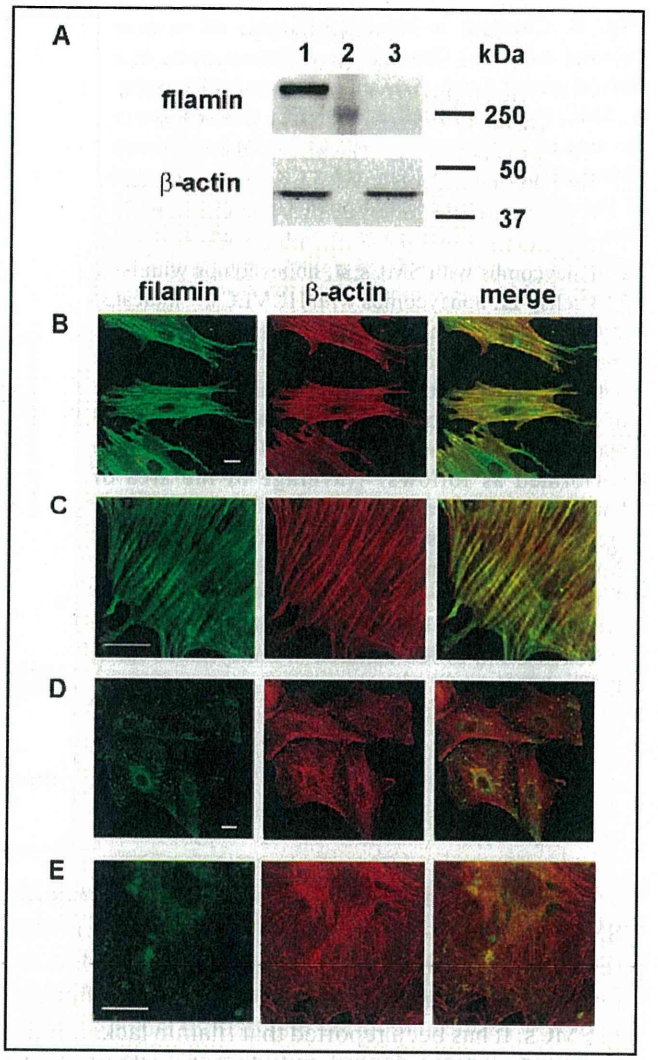

Fig. 7. Expression and localization of filamin and β-actin in ODC-SMCs. The rat ODC gene was stably overexpressed by transfection of a pTracer-CMV plasmid encoding the rat ODC gene into SMCs using electroporation (ODC-SMCs). (A) Western blot analysis of filamin and β-actin in ODC-SMCs. SMCs and ODC-SMCs were cultured on plates to subconfluence. Lane 1, SMC; lane 2, molecular marker; lane 3, ODC-SMC. Localization of filamin and β-actin in SMCs (B, C) and ODC-SMCs (D, E). SMCs and ODC-SMCs were cultured on plates to subconfluence. Green indicates filamin, and red indicates β-actin. Scale bar, 20 μm (B, D); 5 μm (C, E).

Recently, we stably overexpressed the rat ornithine-decarboxylase gene in SMCs (termed 'ODC-SMCs') to study the function of polyamine and ODC-antizyme in SMCs (unpublished data). Interestingly, expression of filamin was dramatically decreased in ODC-SMCs compared to SMCs (Fig. 7A). In ODC-SMCs cultured on plates, β-non-muscle actin filaments were thinner than those of SMCs, although expression of β-actin, as detected

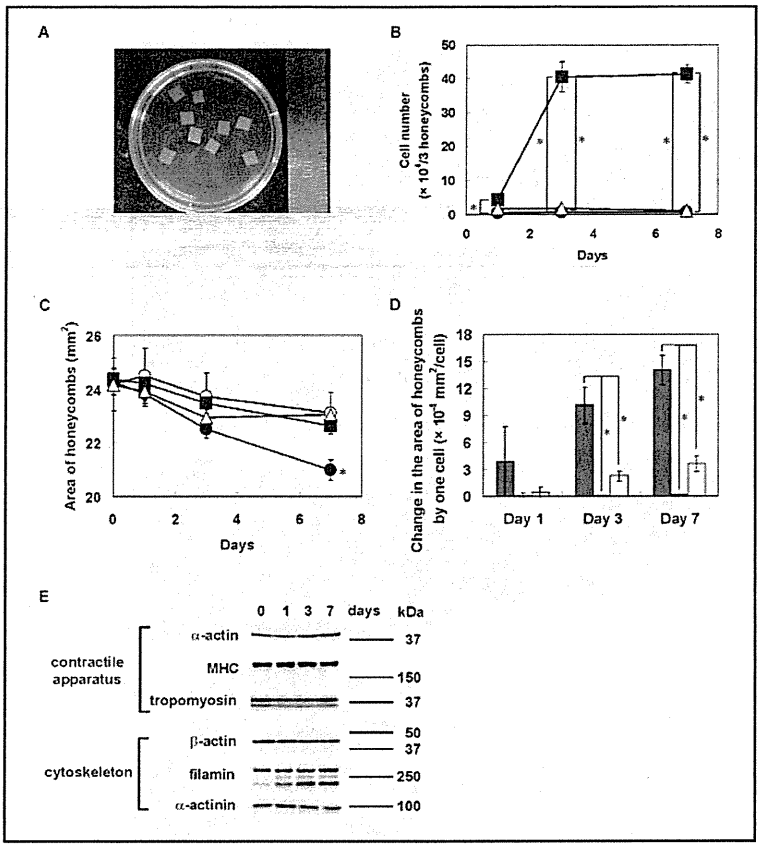

Fig. 8. Changes in honeycomb size of various cultured cells. (A) Observation of honeycombs in a dish (diameter, 6 cm). (B) Cell growth in honeycombs. ●, SMC; ■, NIH 3T3 cell; △, HUVEC. (C) Changes in the area of honeycombs. The size of the honeycomb before culturing with cells was 6 x 6 x 2 mm. A change in the area of the honeycombs (length x width) was observed. o, honeycombs without cells; ●, honeycombs with SMCs; ■, honeycombs with NIH 3T3 cells; △, honeycombs with HUVECs. * indicates $p<0.05$ for the area of honeycombs cultured with a specific cell compared to the area of honeycombs without cells on each day. (D) Reduction of the area of honeycombs by one cell. Closed bar, SMC; shaded bar, NIH3T3 cell; open bar, HUVEC. Calculations were performed as follows: {(average of the area of honeycombs with each type of cells on day 0) – (the area of honeycombs with each type of cells on day 1, 3 or 7)} / the cell number of each type of cell on day 1, 3 or 7. * indicates $p<0.05$. (E) Western blot analysis of proteins involved in the contractile apparatus and cytoskeleton in SMCs cultured in honeycombs for 0, 1, 3 and 7 days.

by western blotting analysis, was almost the same in SMCs and ODC-SMCs (Fig. 7B, C, D and E). These results suggest that the 280 kDa form of filamin is responsible for bundling of β-non-muscle actin filaments in SMCs. It has been reported that filamin lacking a self-association domain cannot induce bundling of actin filaments *in vitro* [13]. These data suggest that degradation of filamin in SMCs cultured in honeycombs induces relative structural weakness of β-non-muscle actin filaments.

Shrinkage of honeycombs used for culturing SMCs

Changes in the size of the honeycombs used for culturing SMCs and other non-contractile cells (NIH3T3 cells and HUVECs) were investigated to determine whether SMCs cultured in honeycombs can contract. SMCs mainly cross perpendicular to the pore of the honeycombs; therefore, it was expected that the height of honeycombs would not be dramatically changed by SMCs. Honeycomb size (length x width) was measured as honeycomb area (Fig. 8A). NIH3T3 cells were attached across the lumens of the honeycombs, while HUVEC were attached to the walls of honeycombs and maintained a round shape, similar to that seen when they are grown on plates. NIH3T3 cells proliferated rapidly in honeycombs, reaching confluence on day 3, while the number of SMCs and HUVEC in the honeycombs did not change (Fig. 8B). The area of the honeycombs was reduced after culturing each cell-type (Fig. 8C). Moreover, the honeycomb areas of SMC cultures were reduced to a greater extent each day than the honeycomb areas of the other two cell-types (Fig. 8D). This suggests that the reduced honeycomb area in SMCs is caused by the tension of SMCs attached across the lumen of the honeycombs or that these SMCs have contractile activity. Expression of proteins involved in both the contractile apparatus and the cytoskeleton were detected in SMCs cultured in honeycombs (Fig. 8E). Expression of α-actin, smooth muscle myosin heavy chain (MHC), and tropomyosin (components of the contractile apparatus) was almost the same in SMCs cultured in honeycombs as in SMCs at day 0. Among components of the cytoskeleton, expression of β-actin and α-actinin were unchanged. SMCs at day 0 expressed the 280 kDa form of filamin, whereas SMCs cultured in honeycombs expressed both the 280 and 180 kDa forms of filamin. In subconfluent SMCs cultured on plates or in SMCs cultured in honeycombs, α- and β-actin were co-localized (Fig. 9), suggesting that the contractile apparatus was

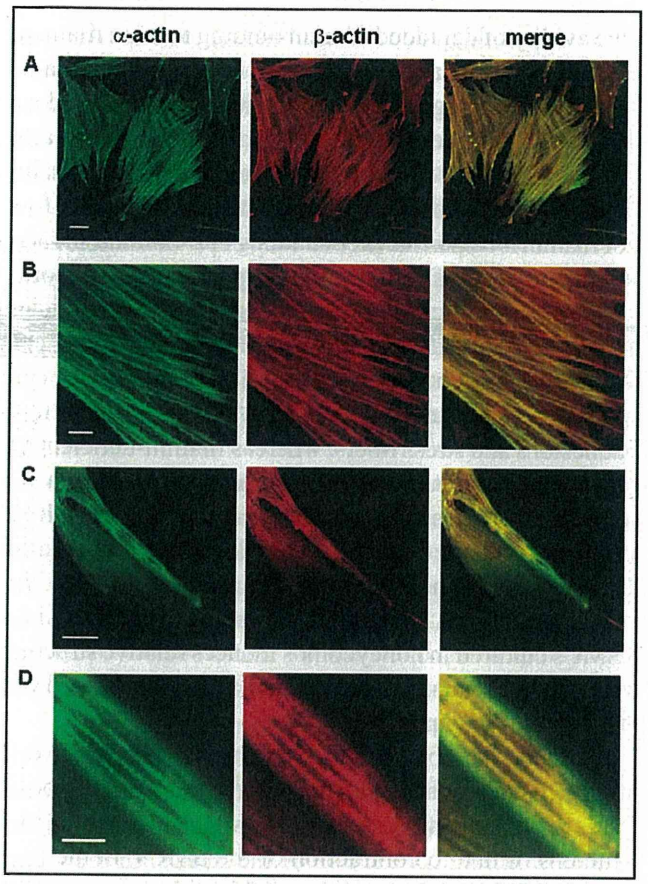

Fig. 9. Localization of α-actin and β-actin in SMCs. (A, B) SMCs were cultured on a plate to subconfluence. (C, D) SMCs were cultured in a honeycomb for 3 days. Green indicates α-actin, and red indicates β-actin. Scale bar, 20 μm (A, C); 5 μm (B, D).

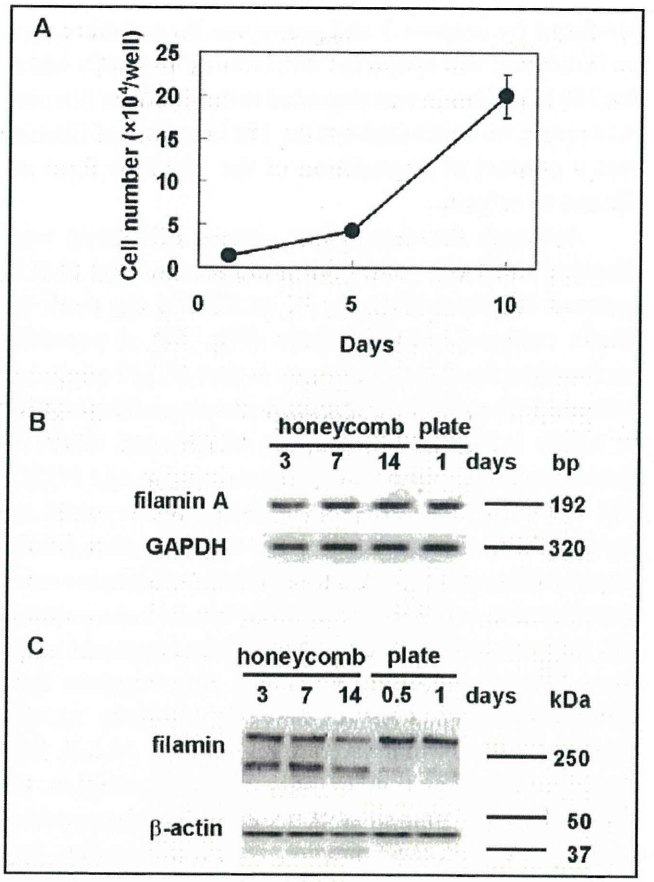

Fig. 10. Continuous expression of filamin mRNA in SMCs under different culture conditions. SMCs were cultured in honeycombs for 14 days and treated with collagenase. Isolated SMCs were plated at 1 x 10^5 cells per well (6-well plate) (A) Cell growth of SMCs cultured on plates after culturing in honeycombs for 14 days. (B) RT-PCR analysis of filamin A. (C) Western blot analysis of filamin.

aligned with β-non-muscle actin filaments. These findings suggest that degradation of filamin reduces the cellular tension maintained by β-non-muscle actin filaments, indicating that SMCs in honeycombs achieve contractility.

Continuous expression of filamin mRNA in SMCs under different culture conditions

SMCs cultured in honeycombs for 14 days immediately started to proliferate when they were transferred to plates (Fig.10A), and their growth rate was almost the same as that of SMCs prepared from media. Filamin A mRNA expression was almost identical in SMCs cultured in honeycombs and on plates (Fig. 10B), and SMCs moved to plates mainly expressed the 280 kDa form of filamin after 12 hours (Fig. 10C). These observations indicate that filamin might contribute to the phenotypic changes seen in SMCs.

Discussion

In this study, we focused on the regulation and role of filamin in SMCs because the molecular weight of filamin changed in SMCs cultured in honeycombs. Our findings indicate that the change in the molecular weight of filamin might be related to rearrangement of the cytoskeletal structure. Finally, our results suggest that SMCs cultured in honeycombs exhibit contraction.

When SMCs were cultured on plates, a 180 kDa form of filamin could be produced by degradation of the 280 kDa form of filamin, and this degradation was almost completely suppressed by the calpain inhibitor ALLN (Fig. 3D). Other enzyme candidates that might also degrade filamin are caspase 3 and granzyme B, two key mediators of apoptosis [25, 26]; however, the pattern of filamin degradation produced by calpain is different from that

produced by caspase 3 and granzyme B, and there was no indication that apoptosis was induced in SMCs when the 280 kDa filamin was degraded to the 180 kDa filamin. As a result, we concluded that the 180 kDa form of filamin was a product of degradation of the 280 kDa form of filamin by calpain.

Although the degradation pattern of filamin was identical in SMCs cultured in honeycombs and SMCs cultured on plates (Figs. 1, 3), ALLN did not work in SMCs cultured in honeycombs (Fig. 3E). A possible explanation for this discrepancy is that ALLN might be released from cells by multidrug resistance protein (MRP)-1, which is known to transport ALLN, and MRP-1-overexpressing cells show distinct resistant to ALLN [27, 28]. Aorta and vascular SMCs have been reported to express MRP-1 abundantly [29]. On the other hand, incorporation activity, such as thymidine and amino acid incorporation into SMCs, was quite low in honeycombs [5], suggesting that SMCs cultured in honeycombs may have difficulty with ALLN uptake. This suggests that SMCs cultured in honeycombs might release ALLN through MRP-1, while barely taking up any ALLN. We therefore suggest that calpain could contribute to degradation of filamin in SMCs cultured in honeycombs in the current study; however, additional studies are needed.

In this study, we identified a clear relationship between calpastatin expression and filamin degradation. Specifically, when calpastatin was expressed in SMCs, the 180 kDa form of filamin was absent, whereas calpastatin was not detected when the 180 kDa form of filamin was present. Although calpastatin is a well-known inhibitor of calpain [30], other studies have shown that calpastatin can be cleaved by μ-calpain and m-calpain *in vitro* when sufficient Ca^{2+} is provided [31, 32]. Despite being cleaved, calpastatin fragments were still potent inhibitors of calpains, though the inhibitory activity was lower [31, 32]. It has been reported that calpain activity is dependent on Ca^{2+}-concentration [23, 33, 34]. When SMCs contract, Ca^{2+} is provided by Ca^{2+} influx from the extracellular space and Ca^{2+} release from the sarcoplasmic reticulum into the cytoplasm [35]. Thus, it can be assumed that an increase in calpain activity induced by higher concentrations of Ca^{2+} might induce cleavage of calpastatin, resulting in calpain-mediated degradation of filamin due to the reduced inhibitory activity of the degraded calpastatin.

Examination of the effects of filamin degradation on the cytoskeleton also yielded interesting data. It is known that degraded filamin can no longer dimerize, and the avidity of degraded filamin binding to actin filaments is reduced compared with that of non-degraded filamin [15]. Our data suggest that degradation of filamin reduces the interaction between filamin and β-non-muscle actin filaments, which results in disruption of their co-localization (Figs 5, 6). Our data also suggest that the 280 kDa form of filamin is responsible for bundling of β-non-muscle actin filaments in SMCs (Fig. 7). This hypothesis is supported by the observation that stress fibers in filamin-deficient human melanoma (M2) cells were compared with A7 cells that were stably reconstituted with filamin [36]. A7 cells spread on fibronectin and formed well-developed focal adhesions and stress fibers, whereas filamin-deficient M2 cells attached, but remained retracted with only a few small focal adhesions and rare short actin bundles. It has also been reported that filamin lacking a self-association domain cannot induce bundling of actin filaments *in vitro* [13]. These data suggest that degradation of filamin in SMCs cultured in honeycombs induces relative structural weakness of β-non-muscle actin filaments compared with the contractile apparatus.

Honeycombs with SMCs shrank significantly over time (Fig. 8). This shrinkage of honeycombs could be the result of the tension within SMCs attached across their lumens or due to contraction of the SMCs. In the latter case, SMCs in honeycombs could achieve contractility because cellular tension maintained by β-non-muscle actin filaments is reduced in these cells due to filamin degradation. The expression levels of α-actin, MHC, tropomyosin, β-actin and α-actinin were almost identical in SMCs cultured on plates and in honeycombs, but filamin in SMCs cultured in honeycombs was degraded (Fig.8E). In subconfluent SMCs cultured on plates, or SMCs cultured in honeycombs, α- and β-actin were co-localized (Fig. 9), which suggests that the contractile apparatus is aligned with β-non-muscle actin filaments. SMCs cultured on plates do not contract under normal culturing conditions [37]. Our results could explain why contraction, which is a result of shortening of the contractile apparatus, was not observed in SMCs cultured on plates. SMCs attach to rigid plastic plates via focal adhesion, and the resulting cytoskeletal tension, which is maintained by β-non-muscle actin filaments, inhibits shortening of the contractile apparatus. In honeycombs, SMCs can achieve contractility because filamin degradation reduces the cytoskeletal tension and allows shortening of the contractile apparatus. Features of dedifferentiated SMCs are large amounts of rough endoplasmic reticulum, free ribosomes and mitochondria, and proliferation in response to PDGF-BB [4, 38]. In our model, the number of

ribosomes and mitochondria present in a certain area of SMCs cultured in honeycombs was almost identical with the amount of ribosomes and mitochondria in aortic SMCs, and the number of ribosomes and mitochondria in a single cell in honeycombs was 20% to 25% of that in SMCs cultured on plates. SMCs cultured in honeycombs also stopped proliferating and did not respond to PDGF-BB anymore [5]. Additionally, SMCs cultured in honeycombs expressed caldesmon heavy chain (a marker protein of mature differentiated SMCs) [5, 6]. Although expression levels of α-actin and MHC (a marker of differentiated SMCs) in SMCs cultured on plates and in honeycombs were almost identical, SMCs in honeycombs might be able to achieve contractility. Based on these results, we believe that SMCs cultured in honeycombs have the functional ability to contract.

When SMCs were released from honeycombs and moved to plates, they immediately began to proliferate (Fig. 10). This response could be regarded as similar in some respects to the change to a proliferative phenotype of SMCs *in vivo*. It is assumed that SMCs can adopt a suitable cytoskeletal structure for their surrounding environment by rearrangement of actin filaments, and filamin can regulate this rearrangement. The results presented herein suggest that constant expression of filamin mRNA and regulation of filamin function by degradation, which is a post-translational regulatory mechanism, might at least partially contribute to the immediate and reversible phenotypic changes in SMCs.

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References

1 Armstrong ML, Warner ED, Connor WE: Regression of coronary atherosclerosis in rhesus monkeys. Circ Res 1970;27:59-67.

2 Corti R, Osende JI, Fallon JT, Fuster V, Mizsei G, Jneid H, Wright SD, Chaplin WF, Badimon JJ: The selective peroxisomal proliferator-activated receptor-gamma agonist has an additive effect on plaque regression in combination with simvastatin in experimental atherosclerosis: *in vivo* study by high-resolution magnetic resonance imaging. J Am Coll Cardiol 2004;43:464-473.

3 Chamley-Campbell J, Campbell GR, Ross R: The smooth muscle cell in culture. Physiol Rev 1979;59:1-61.

4 Chamley-Campbell JH, Campbell GR, Ross R: Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens. J Cell Biol 1981;89:379-383.

5 Ishii I, Tomizawa A, Kawachi H, Suzuki T, Kotani A, Koshushi I, Itoh H, Morisaki N, Bujo H, Saito Y, Ohmori S, Kitada M: Histological and functional analysis of vascular smooth muscle cells in a novel culture system with honeycomb-like structure. Atherosclerosis 2001;158:377-384.

6 Suzuki T, Ishii I, Kotani A, Masuda M, Hirata K, Ueda M, Ogata T, Sakai T, Ariyoshi N, Kitada M: Growth inhibition and differentiation of cultured smooth muscle cells depend on cellular crossbridges across the tubular lumen of type I collagen matrix honeycombs. Microvasc Res 2009;77:143-149.

7 Small JV, Fürst DO, De Mey J: Localization of filamin in smooth muscle. J Cell Biol 1986;102:210-220.

8 North AJ, Gimona M, Lando Z, Small JV: Actin isoform compartments in chicken gizzard smooth muscle cells. J Cell Sci 1994;107:445-455.

9 Small JV, Gimona M: The cytoskeleton of the vertebrate smooth muscle cell. Acta Physiol Scand 1998;164:341-348.

10 Worth NF, Rolfe BE, Song J, Campbell GR: Vascular smooth muscle cell phenotypic modulation in culture is associated with reorganisation of contractile and cytoskeletal proteins. Cell Motil Cytoskeleton 2001;49:130-145.

11 Sjöblom B, Salmazo A, Djinoviæ-Carugo K: Alpha-actinin structure and regulation. Cell Mol Life Sci 2008;65:2688-2701.

12 van der Flier A, Sonnenberg A: Structural and functional aspects of filamins. Biochim Biophys Acta 2001;1538:99-117.

13 Nakamura F, Osborn TM, Hartemink CA, Hartwig JH, Stossel TP: Structural basis of filamin A functions. J Cell Biol 2007;179:1011-1025.

14 Dabrowska R, Goch A, Osiñska H, Szpacenko A, Sosinski J: Dual effect of filamin on actomyosin ATPase activity. J Muscle Res Cell Motil 1985;6:29-42.

15 Gorlin JB, Yamin R, Egan S, Stewart M, Stossel TP, Kwiatkowski DJ, Hartwig JH: Human endothelial actin-binding protein (ABP-280, nonmuscle filamin):a molecular leaf spring. J Cell Biol 1990;111:1089-1105.

16 Huff-Lonergan E, Mitsuhashi T, Beekman DD, Parrish FC Jr, Olson DG, Robson RM: Proteolysis of specific muscle structural proteins by mu-calpain at low pH and temperature is similar to degradation in postmortem bovine muscle. J Anim Sci 1996;74:993-1008.

17 Kwak KB, Chung SS, Kim OM, Kang MS, Ha DB, Chung CH: Increase in the level of m-calpain correlates with the elevated cleavage of filamin during myogenic differentiation of embryonic muscle cells. Biochim Biophys Acta 1993;1175:243-249.

18 Carragher NO, Levkau B, Ross R, Raines EW: Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. J Cell Biol 1999;147:619-630.
19 Chiang W, Greaser ML, Lyons GE: Filamin isogene expression during mouse myogenesis. Dev Dyn 2000;217:99-108.
20 Takafuta T, Wu G, Murphy GF, Shapiro SS: Human beta-filamin is a new protein that interacts with the cytoplasmic tail of glycoprotein Ibalpha. J Biol Chem 1998;273:17531-17538.
21 Chen M, Stracher A: *In situ* phosphorylation of platelet actin-binding protein by cAMP-dependent protein kinase stabilizes it against proteolysis by calpain. J Biol Chem 1989;264:14282-14289.
22 Jay D, Garcia EJ, Lara JE, Medina MA, de la Luz Ibarra M: Determination of a cAMP-dependent protein kinase phosphorylation site in the C-terminal region of human endothelial actin-binding protein. Arch Biochem Biophys 2000;377:80-84.
23 Goll DE, Thompson VF, Li H, Wei W, Cong J: The calpain system. Physiol Rev 2003;83:731-801.
24 Aoyama K, Matsuda T, Aoki N: Characterization of newly identified four isoforms for a putative cytosolic protein tyrosine phosphatase PTP36. Biochem Biophys Res Commun 1999;266:523-531.
25 Umeda T, Kouchi Z, Kawahara H, Tomioka S, Sasagawa N, Maeda T, Sorimachi H, Ishiura S, Suzuki K: Limited proteolysis of filamin is catalyzed by caspase-3 in U937 and Jurkat cells. J Biochem 2001;130:535-542.
26 Browne KA, Johnstone RW, Jans DA, Trapani JA: Filamin (280-kDa actin-binding protein) is a caspase substrate and is also cleaved directly by the cytotoxic T lymphocyte protease granzyme B during apoptosis. J Biol Chem 2000;275:39262-39266.
27 Inoue S, Sharma RC, Schimke RT, Simoni RD: Cellular detoxification of tripeptidyl aldehydes by an aldo-keto reductase. J Biol Chem 1993;268:5894-5898.
28 de Jong MC, Slootstra JW, Scheffer GL, Schroeijers AB, Puijk WC, Dinkelberg R, Kool M, Broxterman HJ, Meloen RH, Scheper RJ: Peptide transport by the multidrug resistance protein MRP1. Cancer Res 2001;61:2552-2557.
29 Mueller CF, Wassmann K, Widder JD, Wassmann S, Chen CH, Keuler B, Kudin A, Kunz WS, Nickenig G: Multidrug resistance protein-1 affects oxidative stress, endothelial dysfunction, and atherogenesis via leukotriene C4 export. Circulation 2008;117:2912-2918.
30 Hanna RA, Campbell RL, Davies PL: Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. Nature 2008;456:409-412.
31 Nakamura M, Inomata M, Imajoh S, Suzuki K, Kawashima S: Fragmentation of an endogenous inhibitor upon complex formation with high- and low-Ca^{2+}-requiring forms of calcium-activated neutral proteases. Biochemistry 1989;28:449-455.
32 Doumit ME, Koohmaraie MJ: Immunoblot analysis of calpastatin degradation: evidence for cleavage by calpain in postmortem muscle. J Anim Sci 1999;77:1467-1473.
33 Edmunds T, Nagainis PA, Sathe SK, Thompson VF, Goll DE: Comparison of the autolyzed and unautolyzed forms of mu- and m-calpain from bovine skeletal muscle. Biochim Biophys Acta 1991;1077:197-208.
34 Arthur JS, Crawford C: Investigation of the interaction of m-calpain with phospholipids: calpain-phospholipid interactions. Biochim Biophys Acta 1996;1293:201-206.
35 Ganitkevich V, Hasse V, Pfitzer G: Ca^{2+}-dependent and Ca^{2+}-independent regulation of smooth muscle contraction. J Muscle Res Cell Motil 2002;23:47-52.
36 Mammoto A, Huang S, Ingber DE: Filamin links cell shape and cytoskeletal structure to Rho regulation by controlling accumulation of p190RhoGAP in lipid rafts. J Cell Sci 2007;120:456-467.
37 Han M, Wen JK, Zheng B, Cheng Y, Zhang C: Serum deprivation results in redifferentiation of human umbilical vascular smooth muscle cells. Am J Physiol Cell Physiol 2006;291:C50-58.
38 Chamley-Campbell JH, Campbell GR: What controls smooth muscle phenotype? Atherosclerosis 1981;40:347-357.