Histol Histopathol (2003) 18: 343-351

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

ß1-integrins co-localize with Na, K-ATPase, epithelial sodium channels (ENaC) and voltage activated calcium channels (VACC) in mechanoreceptor complexes of mouse limb-bud chondrocytes

M. Shakibaei[1] and A. Mobasheri[2]

[1]Institut für Anatomie, Freie Universität Berlin, Berlin, Germany and

[2]Connective Tissue Research Group, Faculty of Veterinary Science, University of Liverpool, Liverpool, United Kingdom

Summary. Interactions between chondrocytes and their extracellular matrix are partly mediated by ß1-integrin receptors. Recent studies have shown that ß1-integrins co-localize with a variety of cytoskeletal complexes, signaling proteins and growth factor receptors. Since mechanosensitive ion channels and integrins have been proposed to participate in skeletal mechanotransduction, in this study, we investigated the possible co-localization of ß1-integrins with two ion channels and a P-type ATPase in mouse limb-bud chondrocytes. The α subunits of Na, K-ATPase, the epithelial sodium channel (ENaC) and the voltage activated calcium channel (VACC) were immunostained in organoid cultures derived from limb-buds of 12-day-old mice using well-characterized antibodies . Indirect immunofluorescence revealed abundant expression of ß1-integrins and each of the selected systems in limb-bud chondrocytes. Two-fluorochrome immunostaining demonstrated that ß1-integrin, Na, K-ATPase, ENaC and VACC co-localize in chondrocytes. Co-imunoprecipitation experiments revealed co-localization and association of integrins with ENaC, VACC and Na, K-ATPase. Cellular responses and signaling cascades initiated by the influx of calcium or sodium through putative mechanosensitive channels may be regulated more effectively if such channels were organized around integrins with receptors, kinases and cytoskeletal complexes clustered about them. The close proximity of ATPase ion pumps such as Na, K-ATPase to chondrocyte mechanoreceptor complexes could facilitate rapid homeostatic responses to the ionic perturbations brought about by activation of mechanically gated cation channels and efficiently regulate the intracellular milieu of chondrocytes.

Offprint requests to: Dr. A. Mobasheri, Department of Veterinary Preclinical Sciences, Faculty of Veterinary Science, University of Liverpool, Liverpool L69 7ZJ, United Kingdom. Fax: +44 151 794 4243. e-mail: a.mobasheri@liverpool.ac.uk

Key words: ß1-integrin, Na, K-ATPase, ENaC, voltage activated calcium channel (VACC), chondrocyte mechanoreceptor complex, co-localization

Introduction

Molecular interactions between chondrocytes and extracellular matrix (ECM) proteins such as type II collagen and fibronectin influence the proliferation and phenotypic differentiation of the cells and morphogenesis of cartilage tissue (von der Mark et al., 1977). Integrins are cell-surface receptors that mediate cell attachment to ECM components and act as molecular conduits for inside-out and outside-in signal transduction in focal adhesion contact sites (Ruoslahti and Pierschbacher, 1987). The ECM in articular cartilage is not only a mechanical framework, but is also important for chondrocyte differentiation, phenotypic stabilization and promotion of chondrocyte survival in the matrix. The integrin-mediated adhesion of chondrocytes to the ECM prevents apoptosis and is important for the continued survival of these cells (Hirsch et al., 1997). Integrins are heterodimers of specific combinations of α and ß subunits that form cell attachment and mechanoreceptor complexes (Wang et al., 1993; Ingber, 1997; Svoboda, 1998). Interactions between the ECM and chondrocytes are partly mediated by the ß1-integrin subfamily of receptors; integrin-mediated adhesion of chondrocytes to type II collagen leads to phosphorylation of cytoskeletal and signaling proteins in focal adhesion sites (Shakibaei, 1995; Shakibaei et al., 1999). Interactions of plasma membrane integrins, associated proteins in mechanoreceptor complexes and subcellular signaling components are an important component of the mechanotransduction pathway (Mobasheri and Martín-Vasallo, 1999; Lee et al., 2000; Salter et al., 2001; Shakibaei and Mobasheri, 2001; Mobasheri et al., 2002a).

It is becoming increasingly apparent that integrins are not passive cell adhesion molecules solely concerned with linking the ECM and the cellular cytoskeleton; integrins also collaborate with a variety of extracellular receptors involved in intracellular biochemical signaling pathways and proteolytic enzymes concerned with cartilage degradation in the ECM. Recent studies have suggested that integrins co-localize with a variety of proteins in chondrocytes including the insulin-like growth factor-I receptor (Shakibaei et al., 1999), caveolins (Schwab et al., 2000), matrix metalloproteases (Schulze-Tanzil et al., 2001) and urokinase-type plasminogen activator receptor (Schwab et al., 2001). Integrins have long been proposed as components of mechanosensory focal adhesion complexes containing calcium-selective ion channels that are involved in transduction of mechanical stimuli in bone, cartilage and other cell types (Pickard, 1994; Duncan, 1995; Salter et al., 1997). There is evidence from other cell systems that integrins (particularly α5ß1 integrin), integrin-linked tyrosine kinases and focal adhesion proteins such as focal adhesion kinase (FAK) regulate L-type calcium channel activity (Wu et al., 2001). Calcium currents mediated by L-type calcium channels have been shown to be integrin-dependent (Chan et al., 2001) and integrins directly modulate the release of calcium from intracellular stores and trigger cytoplasmic calcium waves that propagate throughout the cells bringing about changes in gene expression. It has been proposed that such interactions may represent a novel mechanism for control of calcium influx in vascular smooth muscle and other cell types (Wu et al., 1998, 2001).

Collaboration between integrins, ion channels and ion pumps in chondrocytes may be a pre-requisite event preceding mechanotransduction, cytoskeletal reorganization and more fundamental activities such as chondrocyte differentiation and survival. Since stretch-activated ion channels and transporters have been proposed to play a role in mechanotransduction in chondrocytes (Mobasheri et al., 2002a,b), in this study, we investigated the possible co-localization of integrins with two ion channels and a P-type ATPase pump that have been previously identified in chondrocytes (Mobasheri et al., 1998; Trujillo et al., 1999a,b). A preliminary form of this paper has been published in abstract form (Shakibaei and Mobasheri, 2001).

Materials and methods

Antibodies

Pan antibody against the α subunit of the voltage activated calcium channel (VACC) was purchased from Alomone Labs (Jerusalem, Israel). This polyclonal antiserum which recognizes all α_1 subunits was raised against a purified peptide (C)DNFDYLTRDWSILGPHH LD corresponding to residues 1382-1400 of the α_{1S} subunit of VACC ($CP_{1382-1400}$) and affinity purified by column chromatography using immobilized $CP^{1382-1400}$ (Striessnig et al., 1990a,b; Westenbroek et al., 1992a,b). The polyclonal antibody against the α subunit of the epithelial sodium channel (ENaC) was a gift from Dr. C.M. Canessa (Department of Cellular and Molecular Physiology, Yale University, New Haven, USA) (Duc et al., 1994). This antibody has been used successfully in previous studies to detect αENaC expression in human chondrocytes and human osteoblasts (Trujillo et al., 1999b; Mobasheri and Martín-Vasallo, 1999; Mobasheri et al., 2001). The antibodies to Na, K-ATPase (monoclonal antibodies α6F and α5) were gifts from Dr. D.M. Fambrough (Johns Hopkins University, Baltimore, USA) (Takeyasu et al., 1988; Lebovitz et al., 1989). The antibodies α5 and α6F to Na,K-ATPase were also purchased from the Developmental Studies Hybridoma Bank (DSHB http://www.uiowa.edu/~dshbwww/) which is under the auspices of the NICHD and is maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. A polyclonal antibody to ß1-integrin was obtained from Chemicon International (Temecula, CA, USA). A monoclonal antibody to ß1-integrin was obtained from Coulter (Hialeah, Florida, USA). Texas red-coupled goat anti-rabbit IgG and FITC-coupled goat anti-mouse IgG were obtained from Dianova (Hamburg, Germany). Secondary antibody conjugated to alkaline phosphatase was purchased from Boehringer Mannheim (Germany).

Cartilage organoid culture

A detailed description of the culture technique for the cartilage culture used for the following experiments has been published by Zimmermann et al. (1992) and Shakibaei et al. (1993). Upper and lower limb-buds of 12-day-old mouse embryos were excised, rinsed with Hanks' solution and shaken for 20 min in 0.2% dispase (Boehringer Mannheim, Mannheim, Germany) in Ca^{2+}- and Mg^{2+}-free solution. After the addition of medium, the suspension was homogenized by pipetting and separated from non-dissociated tissue fragments by filtration through a nylon mesh with a pore diameter of 20 μm. For sedimentation of the cells, the suspension was centrifuged at 6000g. Aliquots of 10 μl of this cell suspension (cell density: approximately $2x10^6$ cells) were placed onto a membrane filter at the medium/air interface. The cells were grown in an incubator under 5% CO_2 at 37 °C. The medium (Ham's F-12/DMEM: 50/50, 10% FCS, 1% amino acids, 1% L-glutamine, 50 units/ml streptomycin, 50 units/ml penicillin, and 2.5 μg/ml amphotericin B, 25 μg/ml ascorbic acid) was changed every 3 days.

Transmission electron microscopy

Cultures were fixed in 1% glutaraldehyde plus 1% tannic acid in 0.1 M phosphate buffer, pH 7.4, and post-fixed in 1% OsO_4 in phosphate buffer. After rinsing and dehydration in ethanol, the preparations were embedded in Epon (Plano, Marburg, Germany) and cut with an

Ultracut E ultramicrotome (Reichert) and then the sections were stained with 2% uranyl acetate/lead citrate. The specimens were examined with a transmission electron microscope (Zeiss EM 10).

Immunoprecipitation

Immunoprecipitation was carried out as previously described (Shakibaei et al., 1999). Briefly, organoid cultures were extracted via lysis buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% aprotinin, 4 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM phenylmethyl-sulphonylfluoride (PMSF) on ice for 30 min. The cell extract was centrifuged at 13000 rpm for 30 min and cell debris was removed. Prior to immunoprecipitation, the samples were first pre-cleared by incubation with 25 μl normal rabbit IgG and *Staphylococcus aureus* (*S. aureus*) cells (Sigma, Munich, Germany). Samples were incubated with primary antibodies against ß1 integrins (antibodies were diluted 1:100 in a wash buffer consisting of 0.1% Tween 20, 150 mM NaCl, 50 mM TRIS-HCl pH 7.2, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF) for 2 h at 4 °C, followed by *S. aureus* cells, and the suspensions were shaken for 1 h at 4 °C. Control immunoprecipitations were performed by incubating the samples with normal (non-immune) rabbit IgG-serum alone. *S. aureus* cells were washed 5 times with wash buffer and once with 50 mM TRIS-HCl pH 7.2. To examine whether integrins can interact with αNa, K-ATPase, αENaC and the α subunit of VACC in chondrocytes, co-immunoprecipitation assays were performed. After immunoprecipitation with anti-ß1 integrin antibodies, the samples were probed by immunoblotting with antibodies to αNa, K-ATPase, αENaC and αVACC.

Western blot analysis

Samples were separated by SDS-PAGE (7.5% and 10% gels) under reducing conditions. Proteins were transferred onto nitrocellulose, blocked with 5% (w/v) skimmed milk powder in PBS/0.1% Tween 20 overnight at 4 °C and incubated with primary antibodies (anti-αNa, K-ATPase, α subunit of VACC and αENaC) diluted in blocking buffer for 1 h at RT. Membranes were washed in blocking buffer and then incubated with alkaline phosphatase-conjugated secondary antibody diluted in blocking buffer for 30 min at RT. After that membranes were washed in blocking buffer and finally in 0.1 M TRIS, pH 9.5, containing 0.05 M $MgCl_2$, and 0.1M NaCl. Specific binding was detected using nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (p-toluidine salt; Pierce, Rockford, USA) as substrates. Protein determination was performed using the bicinchoninic acid system (Pierce, Rockford, USA).

Immunofluorescence

The seven-day-old organoid cultures were immersed in O.C.T. embedding material (Bayer, Munich, Germany) and immediately frozen in liquid nitrogen. For double immunolabelling 8 μm-thick sections were fixed in methanol and rinsed in PBS for 3x5 min. Sections probed with monoclonal antibody to Na, K-ATPase were also probed with polyclonal antibodies to ß1-integrin. Cryosections exposed to polyclonal antibodies to ENaC and VACC were co-incubated with monoclonal antibody to ß1-integrin. Incubation with the primary antibodies (anti-αNa, K-ATPase, anti-αENaC and anti-αVACC antibodies, diluted 1:50 in PBS) was for 1 h at RT followed by secondary antibody (either goat-anti-rabbit or goat-anti-mouse immunoglobulin conjugated to FITC or TRITC, diluted 1:50 in PBS) for 1 h at RT. Washing with PBS followed each step and the sections were examined under a fluorescence microscope (Axiophot 100, Zeiss, Germany).

Results

Transmission electron microscopy

Mesenchymal cells isolated from limb-buds of mouse embryos cultivated in organoid culture initially aggregated into blastema-like units. After three days, a cartilage-specific matrix developed in these blastemata. After a culture period of 3-7 days, typical cartilage nodules of variable size could be found. The chondrocytes had oval shapes and produced an extracellular matrix (Fig. 1). The cell membranes exhibited numerous small projections into the extracellular matrix and were in direct contact with the surrounding matrix. The cytoplasm contained a well-developed rough endoplasmic reticulum, a large Golgi apparatus and a large round nucleus (Zimmermann et al., 1992; Shakibaei et al., 1993, 1995).

Integrins co-immunoprecipitate with Na, K-ATPase, ENaC and VACC

To examine a possible association of ß1-integrins with Na, K-ATPase, ENaC and VACC in chondrocytes, we performed co-immunoprecipitation assays in the organoid cultures. Immunoprecipitation of cell lysates with antibodies raised against ß1-integrin followed by immunoblotting with antibodies to the selected transport systems demonstrated whether integrins co-localized with any of them. The cells were lysed, cleared and immunoprecipitated with antibodies to ß1-integrin. The ß1-integrin immunoprecipitates were then immunoblotted with antibodies to the α subunits of Na, K-ATPase, ENaC and VACC (using pan α antibodies). Immunoprecipitation experiments provided evidence for co-localization and association of ß1-integrins with the α subunits of ENaC, VACC and Na, K-ATPase (Fig. 2). We deliberately used pan antibodies capable of detecting all the known α subunits of these three membrane proteins in the fully denatured forms in SDS gels. There was no evidence of non-specific immunoreactivity in the Western blots of ß1 integrin immunoprecipitates and no

proteolytic degradation of protein bands occurred. A 110 kDa protein corresponding to the α subunit of Na, K-ATPase was found in ß1-integrin immunoprecipitates but not in control immunoprecipitates (Fig. 2A). A highly glycosylated (90 kDa) αENaC subunit also co-immunoprecipitated with ß1-integrin (Fig. 2B). A large molecular weight protein migrating around 195-200 kDa corresponding to the α subunit of VACC was also found in ß1-integrin immunoprecipitates (Fig. 2C). This protein was not detectable in control immuno-precipitates. Immunoprecipitation with control antibody (non-immune antiserum) did not produce any specific bands in control experiments (lanes labelled C in Fig. 2). Taken together, these findings demonstrate that Na, K-ATPase, ENaC and VACC associate with ß1-integrin adhesion complexes in the plasma membrane of mouse limb-bud chondrocytes.

ß1-integrin co-localizes with Na, K-ATPase, ENaC and VACC in organoid cultures

To demonstrate the expression and co-localization of ß1-integrin with the transport systems, we used double immunofluorescence (indirect method) using FITC conjugated secondary antibodies to localize ß1- integrin immunostaining and TRITC conjugated secondary antibodies to localize the α subunits of Na, K-ATPase, ENaC and VACC. Immunofluorescence microscopy revealed cell-specific expression of ß1-integrin in all organoid culture cells (Fig. 3). The α subunits of Na, K-ATPase, ENaC and VACC were also abundantly expressed in mouse limb-bud chondrocytes (Fig. 3). Both antigens produced similar immunostaining patterns when examined under low and high power magnifications. Most of the immunostaining observed with antibodies to the transport systems was on the cell surface; there was some intracellular, cytoplasmic staining which is typically seen in these cells with subunits of Na, K-ATPase and ENaC (Mobasheri, 1999; Mobasheri and Martín-Vasallo, 1999). There was no non-specific immunostaining in the ECM (indicated by

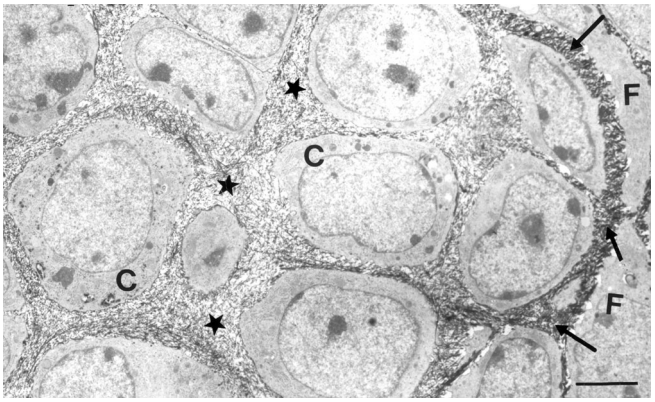

Fig. 1. Electron micrograph of high-density organoid culture from mouse limb bud mesenchymal cells after a cultivation period of 7 days. The transitional zone of perichondrium/cartilage is shown. The flat fibroblast-like cells (F) of the perichondrium are embedded in a dense mass of thick collagen fibrils (as indicated by arrows). Typical chondrocytes (C) are embedded in the cartilage matrix (as indicated by stars). Bar: 2 μm

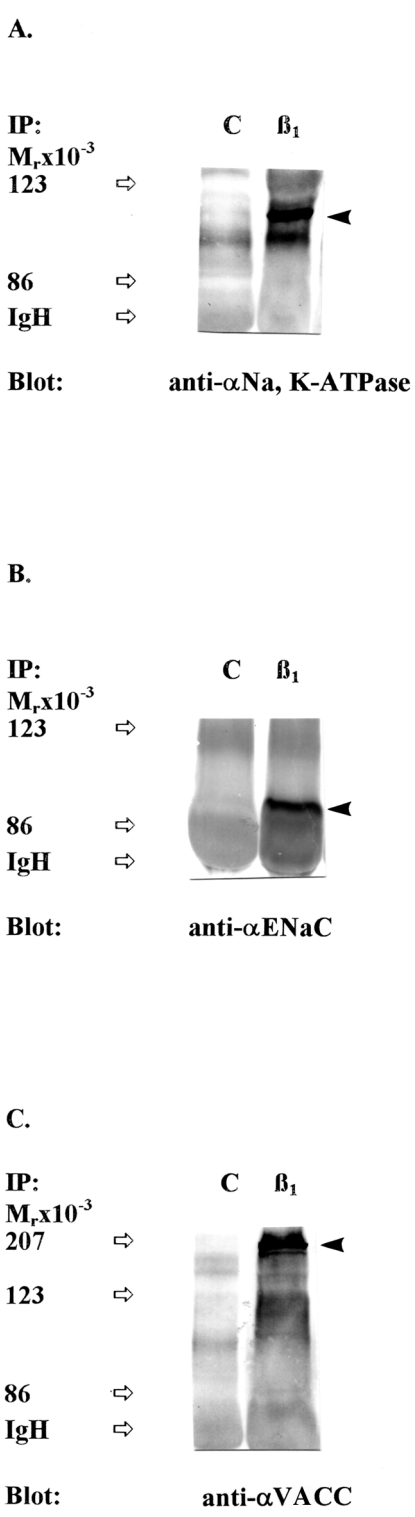

Fig. 2. Co-immunoprecipitation assays and immunoblotting of ß1-integrin, αNa, K-ATPase, αENaC and αVACC. **A**. Immuno-precipitation with anti ß1-integrin antibody followed by immunoblotting with anti-αNa, K-ATPase antibody. **B.** Immuno-precipitation with anti ß1-integrin antibody followed by immunoblotting with anti-αENaC antibody. **C.** Immuno-precipitation with anti ß1-integrin antibody followed by immunoblotting with anti-αVACC antibody. In each panel the left hand Western blot lanes labeled C indicate control immunoprecipitates using non-immune control anti-rabbit immunoglobulin antibody. Visualization was by nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (p-toluidine salt). Molecular weight markers are indicated in kilodaltons.

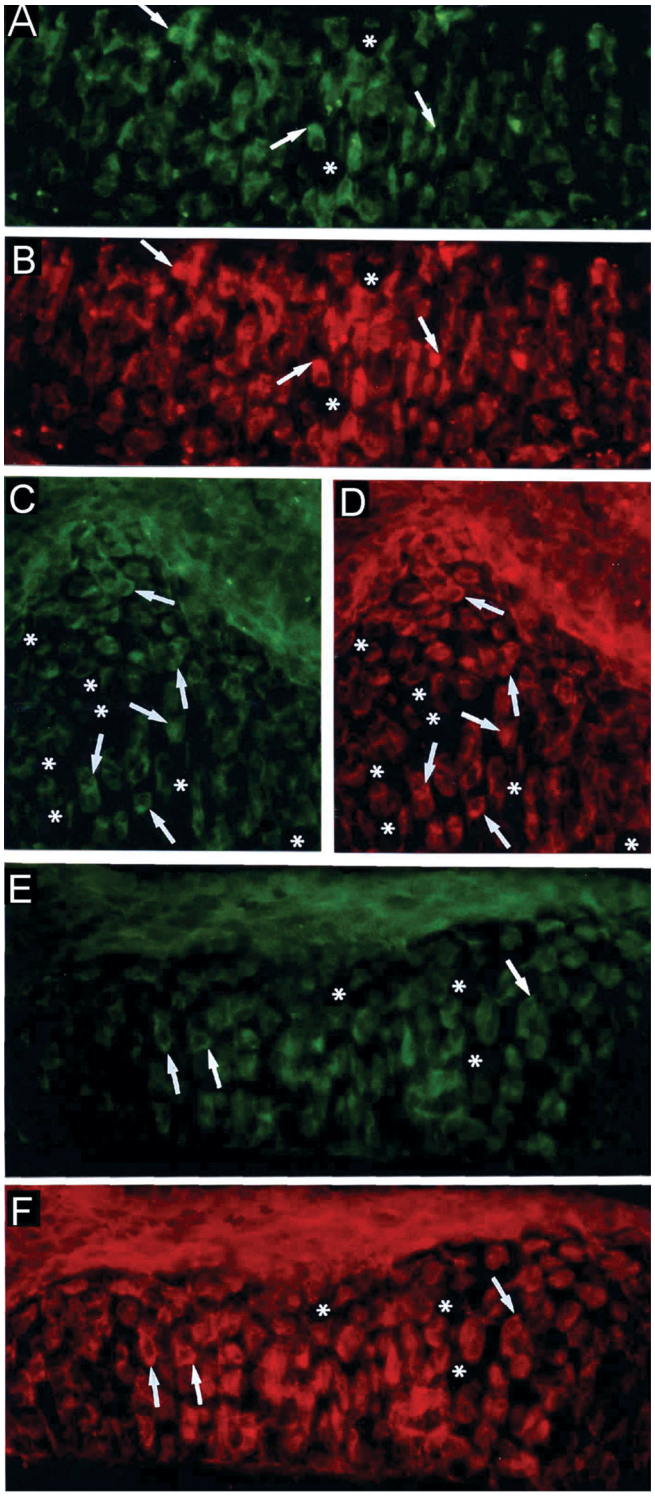

Fig. 3. Immunofluorescence micrographs demonstrating the expression and co-localization of ß1-integrin **(A, C and E)** and the α subunits of Na, K-ATPase **(B)**, ENaC **(D)** and VACC **(F)** in mouse limb-bud chondrocytes grown in organoid culture. Note the high specificity of the cellular immunostaining observed in each case (confined to chondrocytes as indicated by arrows) and the lack of immunostaining in the ECM (indicated by asterisks). Original magnification x 100

asterisks in Fig. 3). Two-fluorochrome immunostaining revealed positive correlation between ß1-integrins, VACC and Na, K-ATPase expression. ENaC and ß1-integrins were also found to co-localize on the cell surface and non-specific staining was not observed in the ECM.

Discussion

The major results of this study indicate that ß1-integrins in chondrocyte adhesion complexes co-localize with two ion channels that have recently been proposed to function as mechanosensitive components of chondrocyte mechanoreceptors (Mobasheri et al., 2002a) and a P-type ion transporting ATPase pump responsible for maintaining intracellular Na^+ and K^+ concentrations and cell homeostasis (Mobasheri et al., 1997a,b). This conclusion is based on the following experimental observations: (i) Indirect immunofluorescence revealed abundant expression of ß1- integrins and all the selected transport systems in mouse limb-bud chondrocytes. Previous observations in bovine chondrocytes suggest an abundance of Na, K-ATPase (Mobasheri et al., 1997a,b, 1998; Mobasheri, 1999) and studies in human chondrocytes have confirmed Na, K-ATPase and αENaC expression (Trujillo et al., 1999a,b). Although there is evidence for VACC activity in chondrocytes, (N-/L-type voltage activated calcium channels; Guilak et al., 1999; Wang et al., 2000a) this is the first time the expression of the α subunit of the VACC has been convincingly demonstrated by immunochemical methods. (ii) Two fluorochrome immunostaining showed co-localization of ß1-integrins and the three selected transport systems in mouse limb-bud chondrocytes in organoid culture. (iii) Furthermore, we could also demonstrate that in chondrocytes, ß1-integrin co-immunoprecipitates with the α subunits of Na, K-ATPase, ENaC and VACC. This is, to our knowledge, the first description that integrins and ion channels or ion pumps are part of the same complexes in chondrocytes.

For non-innervated mechanosensitive cells such as chondrocytes, mechanotransduction may be resolved into several distinct phases: 1) mechano-coupling, the characteristics of the mechanical force applied to the cell via the ECM, 2) biochemical coupling, or the mechanism through which the mechanical signal is transduced into a subcellular biochemical signal, 3) transmission of signal to the nucleus of the effector cell and 4) the effector cell response (i.e. a change in gene expression; Duncan, 1995). In previous studies of mechanotransduction in chondrocytes and fibroblasts it has been demonstrated that Na^+ ions first enter the cells via stretch activated Na^+ channels (possibly through ENaC or voltage activated sodium channels or VASC), resulting in depolarization of the membrane, which is then followed by an elevation in intracellular Ca^{2+} causing hyperpolarization of the membrane (Wright et al., 1996). This opens Ca^{2+} activated K^+ channels resulting in K^+ efflux, in an effort to return the

membrane potential to its original resting value. The inward Na^+ currents mediated by ENaC and/or VASC depolarize the chondrocyte membrane thus beginning a sequence of channel opening events (i.e. VACC, followed by Ca^{2+} activated K^+ channels) elevating intracellular Ca^{2+} and culminating in the propagation of calcium waves that activate subcellular signaling cascades. It has recently been demonstrated that integrins are involved in the secretion of interleukin-4, which acts in an autocrine manner via cytokine receptors and is also involved in the hyperpolarization of the membrane in the chondrocyte mechanotransduction pathway (Millward-Sadler et al., 1999). It has also been suggested that the mechanotransduction pathway is defective in osteoarthritis and abnormalities in such "chondroprotective mechanotransduction pathways" in osteoarthritis may contribute to disease progression (Salter et al., 2001).

The mechanical tension required for activating ion channels in the plasma membrane is most likely transmitted via mechanoreceptor sites containing ß1-integrin, with physical and functional links to the ECM and the cytoskeleton. Functionally, leverage exerted against such adhesion sites will control ion channels in the surrounding fluid mosaic. Reactions initiated by passage of ions through the mechanosensitive channels may be more effectively regulated if such channels were clustered around the mechanoreceptor complexes and if appropriate receptors, kinases, ion pumps, and some key cytoskeletal anchoring sites were in turn clustered about them (Pickard, 1994). Thus the activity of clusters of mechanosensitive channels may contribute to control of cytoskeletal architecture and of signaling proteins within their domain. Furthermore, a variety of regulatory proteins and components of the chondrocyte cytoskeleton may contribute to control of mechanosensitive channel activity.

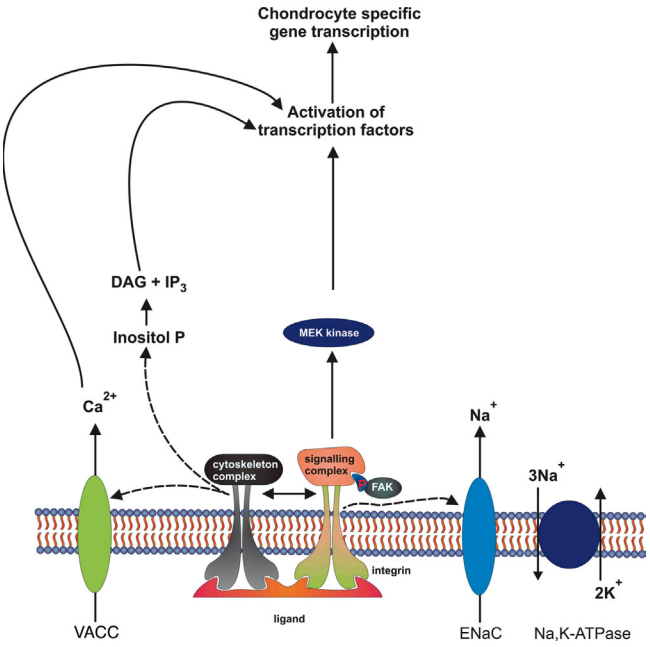

Fig. 4. Proposed model of chondrocyte ß1-integrin/mechanoreceptor complex which implicates the VACC and ENaC ion channels in the chondrocyte mechanotransduction pathway.

Based on the results presented here, the arguments presented by Pickard (1994) and our own hypothetical considerations we propose a chondrocyte mechanoreceptor model, which incorporates ECM macromolecules (collagen type II), ß1-integrins, ENaC and VACC as putative mechanosensitive ion channels (Fig. 4). These ion channels also co-localize with Na, K-ATPase and with ß1-integrins in the chondrocyte plasma membrane and it is highly likely that the mechanosensitive receptors formed by these proteins functionally interact with the cytoskeleton as they do in other cell types, regulating subcellular signal transduction pathways that allow chondrocytes to perceive their physical environment in the ECM (Wang et al., 2001). Needless to say that in addition to mechanoreception, these multi-functional complexes may also be responsible for generating subcellular signals that maintain the chondrocyte phenotype and prevent chondrocyte apoptosis (Shakibaei et al., 1999, 2001).

There is evidence for association between integrins, focal adhesion proteins and ion channels in other cell types (Kwon et al., 2000; Wang et al., 2000b; Chan et al., 2001; Wu et al., 2001). Furthermore, there are indications that ß1-integrins co-localize with G-protein activated inward rectifier potassium (GIRK) channels (McPhee et al., 1998; Ivanina et al., 2000) and voltage activated potassium channels (Kv1.3) (Levite et al., 2000). In T-lymphocytes Kv1.3 channels and ß1-integrins co-immunoprecipitate suggesting that their physical association underlies their functional cooperation (Levite et al., 2000). However, this is the first time ß1-integrins have been shown to co-localize with an ion channel (VACC, ENaC) or ion pump (Na, K-ATPase) in chondrocytes. Therefore, it is possible that in chondrocytes Na, K-ATPase, ENaC and VACC and other ion transport systems may be functionally and physically linked, directly or via hitherto unidentified accessory or linking proteins, to mechanoreceptor complexes in the chondrocyte membrane containing ß1-integrins associated with ECM components such as collagen type II and the intracellular cytoskeleton. There is one report that suggests the α subunit of Na, K-ATPase is a molecular component of an exocytic pathway for basic fibroblast growth factor (FGF-2) (Florkiewicz et al., 1998) and there are experimental papers that suggest the ß2 subunit of Na, K-ATPase functions as a cell adhesion molecule in glia (Antonicek et al., 1987; Pagliusi et al., 1989). We have previously demonstrated that the ß2 subunit of Na, K-ATPase is expressed in chondrocytes (Mobasheri et al., 1997b; Trujillo et al., 1999a). However, there are no reports that experimentally demonstrate an adhesive function for the ß2 subunit in

chondrocytes or its participation in any molecular complex or export function in these cells (Mobasheri et al., 2000). It is tempting to speculate that co-localization of these ion transporters with ß1-integrins in close proximity to ECM proteins permits integrins to modulate these systems following mechanical loading, more specifically deformation of the pericellular matrix. Since deformation induced Na^+ and Ca^{2+} currents have been observed in chondrocytes and osteoblasts following stimulation by mechanical load (Sugimoto et al., 1996; Wright et al., 1996; Kizer et al., 1997), the expression and integrin co-localization of VACC and ENaC in chondrocytes makes them ideal candidates as mechanosensitive ion channels. Collaboration between integrins, growth factor receptors, ion channels and ion transporters in chondrocytes may be a pre-requisite event preceding mechanotransduction, cytoskeletal reorganization and more fundamental activities such as chondrocyte differentiation and survival (Mobasheri and Martín-Vasallo, 1999; Mobasheri, 2002; Mobasheri et al., 2002a). Indeed, cellular responses initiated by calcium or sodium influx through putative mechanosensitive channels may be regulated more effectively if such cation channels were organized around integrins with appropriate receptors, kinases, ion pumps and cytoskeletal complexes clustered in the vicinity (Pickard, 1994). Such structural organization would efficiently streamline the downstream signaling pathways by activated ion channels. The exact sequence of events from integrin activation to mechanosensitive ion channel regulation is unknown. Future studies are required to investigate the role of integrin-mediated signaling cascades in the activity and cell surface expression of ion channels (i.e ENaC, VACC), ATPase ion pumps (i.e. Na, K-ATPase) and other plasma membrane proteins (Mobasheri et al., 2002b) in normal and degenerate articular cartilage.

Acknowledgements. We are grateful to Professor Cecilia M. Canessa for polyclonal antibodies to αENaC. This work was supported by the Teaching Company Directorate (TCS Scheme), the Wellcome Trust (U.K.), the University of Liverpool Research Development Fund (grants 4082 and 4084) and the Deutsche Forschungsgemeinschaft (DFG grant Sh 48/2-4, Sh/2-5).

References

Antonicek H., Persohn E. and Schachner M. (1987). Biochemical and functional characterization of a novel neuron-glia adhesion molecule that is involved in neuronal migration. J. Cell Biol. 104, 1587-1595.

Chan W.L., Holstein-Rathlou N.H. and Yip K.P. (2001). Integrin mobilizes intracellular Ca^{2+} in renal vascular smooth muscle cells. Am. J. Physiol. Cell Physiol. 280, C593-C603.

Duc C., Farman N., Canessa C.M., Bonvalet J.P. and Rossier B.C. (1994). Cell specific expression of epithelial sodium channel α, ß, and γ subunits in aldosterone responsive epithelia from the rat: localization by *in situ* hybridization and immunocytochemistry. J. Cell Biol. 127, 1907-1921.

Duncan R.L. (1995). Transduction of mechanical strain in bone. ASGSB Bull. 8, 49-62.

Florkiewicz R.Z., Anchin J. and Baird A. (1998). The inhibition of fibroblast growth factor-2 export by cardenolides implies a novel function for the catalytic subunit of Na^+,K^+-ATPase. J. Biol. Chem. 273, 544-551.

Guilak F., Zell R.A., Erickson G.R., Grande D.A., Rubin C.T., McLeod K.J. and Donahue H.J. (1999). Mechanically induced calcium waves in articular chondrocytes are inhibited by gadolinium and amiloride. J. Orthop. Res. 17, 421-429.

Hirsch M.S., Lunsford L.E., Trinkaus-Randall V. and Svoboda K.K. (1997). Chondrocyte survival and differentiation *in situ* are integrin mediated. Dev. Dyn. 210, 249-263.

Ingber D.E. (1997). Integrins, tensegrity, and mechanotransduction. Gravit. Space Biol. Bull. 10, 49-55.

Ivanina T., Neusch C., Li Y.X., Tong Y., Labarca C., Mosher D.F. and Lester H.A. (2000). Expression of GIRK (Kir3.1/Kir3.4) channels in mouse fibroblast cells with and without ß1 integrins. FEBS Lett. 466, 327-332.

Kizer N., Guo X.L. and Hruska K. (1997). Reconstitution of stretch-activated cation channels by expression of the α-subunit of the epithelial sodium channel cloned from osteoblasts. Proc. Natl. Acad. Sci. USA 94, 1013-1018.

Kwon M.S., Park C.S., Choi K., Ahnn J., Kim J.I., Eom S.H., Kaufman S.J. and Song W.K. (2000). Calreticulin couples calcium release and calcium influx in integrin-mediated calcium signaling. Mol. Biol. Cell. 11, 1433-1443.

Lebovitz R.M., Takeyasu K. and Fambrough D.M. (1989). Molecular characterization and expression of the (Na^+ + K^+)-ATPase α-subunit in *Drosophila melanogaster*. EMBO J. 8, 193-202.

Lee H.S., Millward-Sadler S.J., Wright M.O., Nuki G. and Salter D.M. (2000). Integrin and mechanosensitive ion channel-dependent tyrosine phosphorylation of focal adhesion proteins and ß-catenin in human articular chondrocytes after mechanical stimulation. J. Bone Miner. Res. 15, 1501-1509.

Levite M., Cahalon L., Peretz A., Hershkoviz R., Sobko A., Ariel A., Desai R., Attali B. and Lider O. (2000). Extracellular $K(^+)$ and opening of voltage-gated potassium channels activate T cell integrin function: physical and functional association between Kv1.3 channels and ß1 integrins. J. Exp. Med. 191, 1167-1176.

McPhee J.C., Dang Y.L., Davidson N. and Lester H.A. (1998). Evidence for a functional interaction between integrins and G protein-activated inward rectifier K1 channels. J. Biol. Chem. 273, 34696-34702.

Millward-Sadler S.J., Wright M.O., Lee H., Nishida K., Caldwell H., Nuki G. and Salter D.M. (1999). Integrin-regulated secretion of interleukin 4: A novel pathway of mechanotransduction in human articular chondrocytes. J. Cell Biol. 145, 183-189.

Mobasheri A. (1999). Brefeldin A influences the cell plasma membrane abundance and intracellular pools of low and high ouabain affinity Na^+, K^+-ATPase α subunit isoforms in articular chondrocytes. Histol. Histopathol. 14, 427-438.

Mobasheri A. (2002). Role of chondrocyte death and hypocellularity in ageing human articular cartilage and the pathogenesis of osteoarthritis. Med. Hypoth. 58, 193-197.

Mobasheri A. and Martín-Vasallo P. (1999). Epithelial sodium channels in skeletal cells; a role in mechanotransduction? Cell Biol. Int. 23, 237-240.

Mobasheri A., Hall A.C., Urban J.P., France S.J. and Smith A.L. (1997a). Immunologic and autoradiographic localization of the Na^+,

K(+)-ATPase in articular cartilage: upregulation in response to changes in extracellular Na^+ concentration. Int. J. Biochem. Cell Biol. 29, 649-657.

Mobasheri A., Errington R.J., Golding S., Hall A.C. and Urban J.P. (1997b). Characterization of the Na^+, K(+)-ATPase in isolated bovine articular chondrocytes; molecular evidence for multiple α and ß isoforms. Cell Biol. Int. 21, 201-212.

Mobasheri A., Mobasheri R., Francis M.J., Trujillo E., Alvarez de la Rosa D. and Martín-Vasallo P. (1998). Ion transport in chondrocytes: membrane transporters involved in intracellular ion homeostasis and the regulation of cell volume, free [Ca^{2+}] and pH. Histol. Histopathol. 13, 893-910.

Mobasheri A., Avila J., Cozar-Castellano I., Brownleader M.D., Trevan M., Francis M.J., Lamb J.F. and Martín-Vasallo P. (2000). Na^+, K^+-ATPase isozyme diversity; comparative biochemistry and physiological implications of novel functional interactions. Biosci. Rep. 20, 51-91.

Mobasheri A., Pocock A.E., Trujillo E., Ferraz I., Martín-Vasallo P. and Francis M.J. (2001). Detection of mRNA and protein of the α subunit of the epithelial sodium channel (ENaC) in human osteoblasts and human ACL cells. J. Physiol. (Lond.) 535P, 4P-5P (Abstract).

Mobasheri A., Carter S.D., Martín-Vasallo P. and Shakibaei M. (2002a). Integrins and stretch activated ion channels; putative components of functional cell surface mechanoreceptors in articular chondrocytes. Cell Biol. Int. 26, 1-18.

Mobasheri A., Vannucci S.J., Bondy C.A., Carter S.D., Innes J.F., Arteaga M.F., Trujillo E., Ferraz I., Shakibaei M. and Martín-Vasallo P. (2002b). Glucose transport and metabolism in chondrocytes: a key to understanding chondrogenesis, skeletal development and cartilage degradation in osteoarthritis. Histol. Histopathol. 17, 1239-1267.

Pagliusi S., Antonicek H., Gloor S., Frank R., Moos M. and Schachner M. (1989). Identification of a cDNA clone specific for the neural cell adhesion molecule AMOG. J. Neurosci. Res. 22, 113-119.

Pickard B.G. (1994). Contemplating the plasmalemmal control center model. Protoplasma. 182, 1-9.

Ruoslahti E. and Pierschbacher M.D. (1987). New perspectives in cell adhesion: RGD and integrins. Science 238, 491-497.

Salter D.M., Robb J.E. and Wright M.O. (1997). Electrophysiological responses of human bone cells to mechanical stimulation: evidence for specific integrin function in mechanotransduction. J. Bone Miner. Res. 12, 1133-1141.

Salter D.M., Millward-Sadler S.J., Nuki G. and Wright M.O. (2001). Integrin-interleukin-4 mechanotransduction pathways in human chondrocytes. Clin. Orthop. 391 Suppl, S49-S60.

Schulze-Tanzil G., de Souza P., Merker H.J. and Shakibaei M. (2001). Co-localization of integrins and matrix metalloproteinases in the extracellular matrix of chondrocyte cultures. Histol. Histopathol. 16, 1081-1089.

Schwab W., Kasper M., Gavlik J.M., Schulze E., Funk R.H. and Shakibaei M. (2000). Characterization of caveolins from human knee joint cartilage: expression of caveolin-1, -2, and -3 in chondrocytes and association with integrin ß1. Histochem. Cell Biol. 113, 221-225.

Schwab W., Gavlik J.M., Beichler T., Funk R.H., Albrecht S., Magdolen V., Luther T., Kasper M. and Shakibaei M. (2001). Expression of the urokinase-type plasminogen activator receptor in human articular chondrocytes: association with caveolin and ß1-integrin. Histochem. Cell Biol. 115, 317-323.

Shakibaei M. (1995). Integrin expression on epiphyseal mouse chondrocytes in monolayer culture. Histol. Histopathol. 10, 339-349.

Shakibaei M. and Mobasheri A. (2001). Sodium potassium pumps, epithelial sodium channels and voltage gated calcium channels colocalize with ß1-integrins in mouse limb-bud chondrocytes in organoid culture. Int. J. Exp. Pathol. 82, A19 (abstract).

Shakibaei M., Abou-Rebyeh H. and Merker H.J. (1993). Integrins in ageing cartilage tissue *in vitro*. Histol. Histopathol. 8, 715-723.

Shakibaei M., Zimmermann B. and Merker H.J. (1995). Changes in integrin expression during chondrogenesis *in vitro*: an immunomorphological study. J. Histochem. Cytochem. 43, 1061-1069.

Shakibaei M., John T., De Souza P., Rahmanzadeh R. and Merker H.J. (1999). Signal transduction by ß1 integrin receptors in human chondrocytes *in vitro*: collaboration with the insulin-like growth factor-I receptor. Biochem. J. 342, 615-623.

Shakibaei M., Schulze-Tanzil G., de Souza P., John T., Rahmanzadeh M., Rahmanzadeh R. and Merker H.J. (2001). Inhibition of mitogen-activated protein kinase kinase induces apoptosis of human chondrocytes. J. Biol. Chem. 276, 13289-13294.

Striessnig J., Glossmann H. and Catterall W.A. (1990a). Identification of a phenylalkylamine binding region within the α1 subunit of skeletal muscle Ca^{2+} channels. Proc. Natl. Acad. Sci. USA 87, 9108-9112.

Striessnig J., Scheffauer F., Mitterdorfer J., Schirmer M. and Glossmann H. (1990b). Identification of the benzothiazepine-binding polypeptide of skeletal muscle calcium channels with (+)-cis-azidodiltiazem and anti-ligand antibodies. J. Biol. Chem. 265, 363-370.

Sugimoto T., Yoshino M., Nagao M., Ishii S. and Yabu H. (1996). Voltage-gated ionic channels in cultured rabbit articular chondrocytes. Comp. Biochem. Physiol. C (Pharmacol. Toxicol. Endocrinol.) 115, 223-232.

Svoboda K.K. (1998). Chondrocyte-matrix attachment complexes mediate survival and differentiation. Microsc. Res. Tech. 43, 111-122.

Takeyasu K., Tamkun M.M., Renaud K.J. and Fambrough D.M. (1988). Ouabain-sensitive (Na^+ + K^+)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the α-subunit of an avian sodium pump. J. Biol. Chem. 263, 4347-4354.

Trujillo E., Alvarez de la Rosa D., Mobasheri A., Avila J., Gonzalez T. and Martín-Vasallo P. (1999a). Sodium transport systems in human chondrocytes. I. Morphological and functional expression of the Na^+, K^+ ATPase α and ß subunit isoforms in healthy and arthritic chondrocytes. Histol. Histopathol. 14, 1011-1022.

Trujillo E., Alvarez de la Rosa D., Mobasheri A., Gonzalez T., Canessa C.M. and Martín-Vasallo P. (1999b). Sodium transport systems in human chondrocytes. II. Expression of ENaC, $Na^+/K^+/2Cl$ cotransporter and Na^+/H^+ exchangers in healthy and arthritic chondrocytes. Histol. Histopathol. 14, 1023-1031.

von der Mark K., Gauss V., von der Mark H. and Muller P. (1977). Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. Nature 267, 531-532.

Wang N., Butler J.P. and Ingber D.E. (1993). Mechanotransduction across the cell surface and through the cytoskeleton. Science 260, 1124-1127.

Wang X.T., Nagaba S., Nagaba Y., Leung S.W., Wang J., Qiu W., Zhao P.L. and Guggino S.E. (2000a). Cardiac L-type calcium channel α1-subunit is increased by cyclic adenosine monophosphate: messenger RNA and protein expression in intact bone. J. Bone

Miner. Res. 15, 1275-1285.

Wang Y.G., Samarel A.M. and Lipsius S.L. (2000b). Laminin acts via ß1 integrin signaling to alter cholinergic regulation of L-type Ca^{2+} current in cat atrial myocytes. J. Physiol. (Lond.). 526, 57-68.

Wang N., Naruse K., Stamenovic D., Fredberg J.J., Mijailovich S.M., Tolic-Norrelykke I.M., Polte T., Mannix R. and Ingber D.E. (2001). Mechanical behavior in living cells consistent with the tensegrity model. Proc. Natl. Acad. Sci. USA 98, 7765-7770.

Westenbroek R.E., Hell J.W., Warner C., Dubel S.J., Snutch T.P. and Catterall W.A. (1992a). Biochemical properties and subcellular distribution of an N-type calcium channel α1 subunit. Neuron 9, 1099-1115.

Westenbroek R.E., Sakurai T., Elliott E.M., Hell J.W., Starr T.V., Snutch T.P. and Catterall W.A. (1992b). Immunochemical identification and subcellular distribution of the α1A subunits of brain calcium channels. J. Neurosci. 15, 6403-6418.

Wright M., Jobanputra P., Bavington C., Salter D.M. and Nuki G. (1996). Effects of intermittent pressure-induced strain on the electrophysiology of cultured human chondrocytes: evidence for the presence of stretch-activated membrane ion channels. Clin. Sci. 90, 61-71.

Wu X., Mogford J.E., Platts S.H., Davis G.E., Meininger G.A. and Davis M.J. (1998). Modulation of calcium current in arteriolar smooth muscle by αv ß3 and α5 ß1 integrin ligands. J. Cell Biol. 143, 241-252.

Wu X., Davis G.E., Meininger G.A., Wilson E. and Davis M.J. (2001). Regulation of the L-type calcium channel by α5 ß1 integrin requires signaling between focal adhesion proteins. J. Biol. Chem. 276, 30285-30292.

Zimmermann B., Schröter-Kermani C., Shakibaei M. and Merker H-J. (1992). Chondrogenesis, cartilage maturation and transformation of chondrocytes in organoid culture of mouse limb bud mesodermal cells. Eur. Arch. Biol. 103, 93-111.

Accepted October 30, 2002