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Expression of Meltrin-α mRNA Is Not Restricted to Fusagenic Cells

Heather A. Harris,* Richard J. Murrills, and Barry S. Komm

Women's Health Research Institute, Wyeth-Ayerst Research, Radnor, Pennsylvania 19087

Abstract Meltrin-α is a myoblast gene product reported to be required for cell fusion [Yagami-Hiromasa et al. (1995): Nature 377:652–656]. Because Northern blots revealed expression only in muscle and bone, the suggestion was made that meltrin-α is expressed exclusively by fusagenic cells in these tissues (myoblast and osteoclast). We studied expression of meltrin-α mRNA in a panel of tissues and cell lines using the polymerase chain reaction and found it widely expressed. Meltrin-α mRNA was readily detected in the osteoblast, the most abundant cell type in bone. In situ hybridization analysis on sections of neonatal mice revealed high levels of expression in the trabecular meshwork of long bones, the basal regions of the dermis and its underlying mesenchyme. We conclude that expression of meltrin-α mRNA is not restricted to fusagenic cells and that, in bone, the osteoblast is the major source. J. Cell. Biochem. 67:136–142, 1997.

Key words: Meltrin-α; ADAM; osteoblast; cell fusion

Although most cells routinely divide to make two, rarely do two cells come together to make one. When cell fusion does occur, it is generally under very controlled circumstances such as fertilization, placenta formation, osteoclastogenesis, and myotube formation. Perhaps cell fusion is restricted to a few cell types because fusion between inappropriate partners would undermine the specialization conferred upon cells after differentiation. Although the biophysics of virus–cell fusion is well studied [e.g., Rodriguez et al., 1996; Durrer et al., 1996], the molecular mechanisms controlling animal cell fusion are only recently becoming elucidated. Sperm–egg fusion has been a favorable model to develop hypotheses about these mechanisms.

Fertilin is a sperm surface heterodimeric glycoprotein strongly implicated in mediating sperm–egg fusion [Blobel et al., 1992; Wolfsberg et al., 1993]. The deduced amino acid sequence of fertilin-α and fertilin-β reveals two features key to its function: a disintegrin domain on the β-subunit and a putative fusion peptide on the α subunit [Blobel et al., 1992; Myles et al., 1994]. In addition to these features, both subunits share homology with metalloproteases (although catalytic activity has not been demonstrated).

Molecular biology techniques have allowed identification of a growing number of proteins with disintegrin and metalloprotease domains, and these proteins are grouped into the ADAM family [reviewed by Wolfsberg et al., 1995]. The biology of most ADAMs is poorly understood; however, two recent reports seek to use the fertilin model to understand cell fusion in other systems [Yagami-Hiromasa et al., 1995; Podbilewicz, 1996].

Yagami-Hiromasa et al. [1995] used degenerate primers to fertilin in an attempt to isolate homologous molecules in myoblasts, which fuse during early development to make syncitial myotubes. Three mouse cDNAs were cloned: Meltrin-α-β-γ. Meltrin-α is a member of the ADAM family and may contain a fusion peptide. By Northern blot analysis, expression of meltrin-α and β was detected in developing muscle and in embryonic and adult bone.

Yagami-Hiromasa et al. [1995] propose that meltrin-α is involved in myoblast fusion. Evidence for this hypothesis comes from the observation that the meltrin-α message appears in C2 cells near the temporal onset of differentiation. In addition, expression of antisense RNA using the predicted active (short) form of meltrin-α suppressed fusion in the C2 muscle cell line and its overexpression facilitated fusion. Since bone also contains multinucleated

*Correspondence to: Dr. Heather Harris, Women's Health Research Institute, Wyeth-Ayerst Research, 145 King of Prussia Rd., Radnor PA 19087. E-mail: harrish@war.wyeth.com

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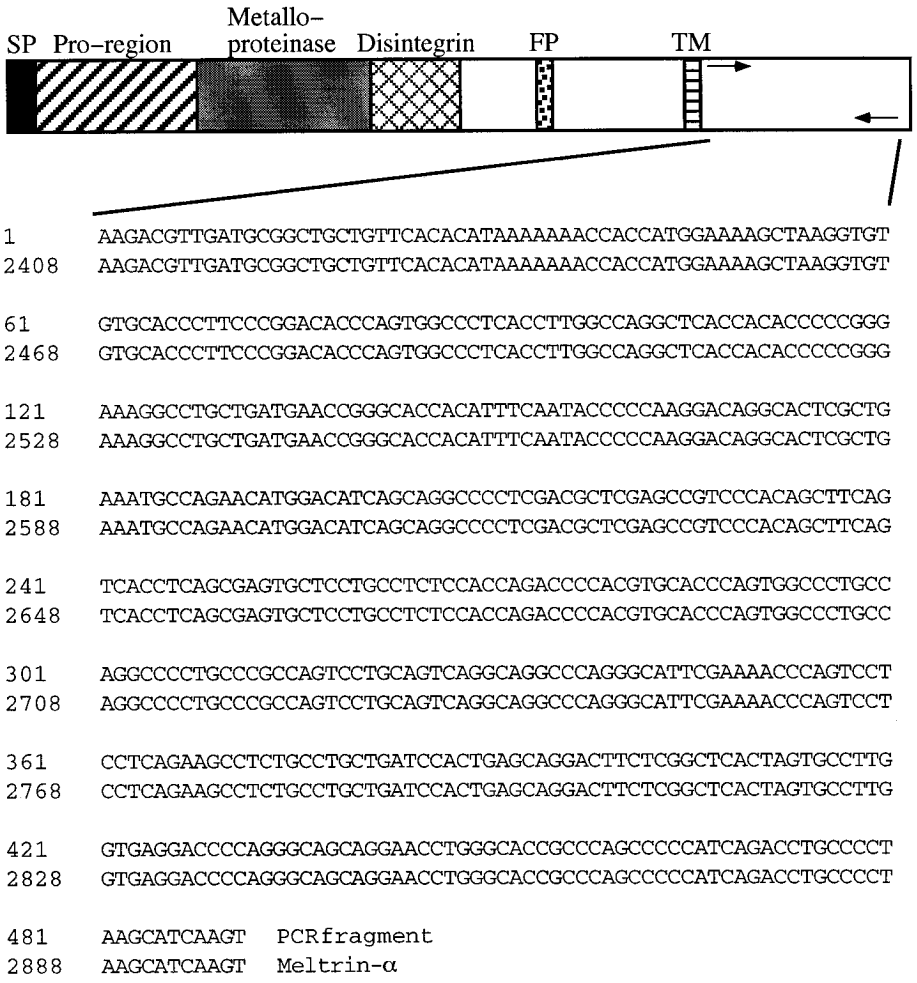

Fig. 1. Schematic diagram of the predicted domain structure of meltrin-α [after Yagami-Hiromasa et al., 1995] illustrating location of primers. SP, signal peptide; FP, putative fusion peptide; TM, transmembrane domain. PCR product was sequenced and was identical to published sequence.

cells (osteoclasts), the authors suggest that the osteoclast may be the source of meltrin-α and β in bone and that meltrin-α may play a role in osteoclast fusion. A recent abstract [Mocharia et al., 1996] also proposes that meltrin-α is involved in osteoclast formation. Evidence cited includes induction of meltrin-α (but not meltrin-β) by vitamin D_3 in mouse bone marrow cultures and in situ reverse transcriptase-polymerase chain reaction (RT-PCR) demonstrating expression in macrophage-derived giant cells.

Intrigued by its apparent restricted distribution, we considered meltrin-α as a possible therapeutic target for osteoporosis: If osteoclast precursor fusion could be inhibited, perhaps bone resorption would be reduced. We tested the hypothesis that meltrin-α was expressed

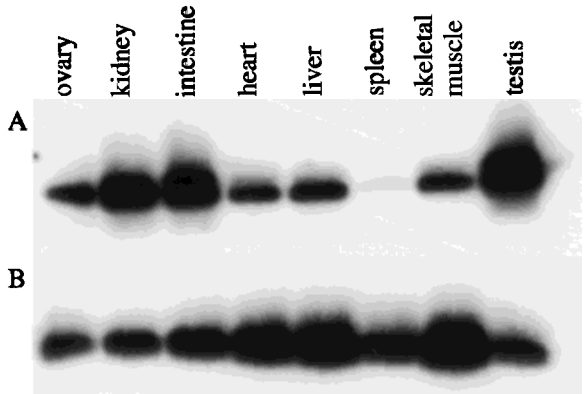

Fig. 2. RT-PCR amplification in various rat tissues. **A:** Meltrin-α. **B:** GAPDH. Different amounts of input RNA were used: ovary, 1.5 μg; kidney, 1.2 μg; intestine, 3.2 μg; heart, 1.6 μg; liver, 2.45 μg; spleen, 3.1 μg; skeletal muscle, 2.0 μg; testis, 1.7 μg. No product resulted from reactions not containing reverse transcriptase.

only by osteoclasts and neonatal myoblasts by using RT-PCR to look at gene expression in a panel of tissues and by in situ hybridization in newborn mice. We find meltrin-α expressed in a wide variety of tissues and that the osteoblast, rather than the osteoclast, is a particularly rich source.

MATERIALS AND METHODS

RT-PCR

Total RNA was prepared from a variety of rodent tissues by careful excision and freezing on dry ice. TRIzol® (Gibco BRL) reagent was used to prepare total RNA according to manufacturer's directions. Osteoblast RNA from cultured fetal rat calvaria was a gift from Drs. Gary Stein and Jane Lian (University of Massachusetts Medical Center). Prior to RT-PCR, all samples were treated with DNase I and RNA recovered by phenol/chloroform extraction and alcohol precipitation.

Primers were designed to amplify a 491-base pair (bp) product from nucleotides 2408–2898 of the published sequence, which corresponds to the cytoplasmic tail of the protein product (Fig. 1). The reverse primer was 5′ ACTTGATGCTTAGGGGCAGGTC, and the forward primer was 5′ AAGACGTTGATGCGGCTGCTGTTC. The National Center for Biotechnology Information's nucleotide database was searched with both primer sequences to ensure little or no homology with other known sequences. The reverse transcriptase reaction was performed for 45 min at 42°C in 20 µl of 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM dNTP mix containing 25 pmol reverse primer, 5 units RNase inhibitor, and 200 units SuperScript™ II reverse transcriptase (Gibco BRL). After heat inactivating the reverse transcriptase, PCR was performed in 100 µl of 26 mM Tris–HCl pH 8.4, 55 mM KCl, 2.4 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM dNTP mix, 25 pmol forward primer, and 2.5U *Taq* DNA polymerase as follows: [45 s at 95°C, 45 s at 66°C, 45 s at 72°C] × 20 cycles, 10 min at 72°C. Control reactions with and without reverse transcriptase were performed in parallel using 25 pmol of primers designed to amplify glyceraldehyde-3-phosphate dehydrogenase. PCR products were separated on an agarose gel and transferred by capillary action to nylon membranes. Blots were hybridized with a ^{32}P-labeled oligonucleotide internal to the primers (bases 2571–2500 for meltrin-α) using Rapid-hyb buffer (Amersham) at 42°C and washed extensively with 30 mM NaCl, 3 mM sodium citrate, 1% (w/v) sodium dodecyl sulfate (SDS) before exposure to film.

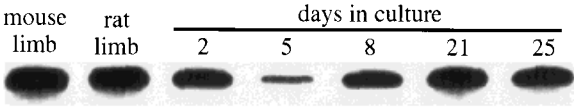

Fig. 3. RT-PCR amplification of meltrin-α. **Lanes 3–7,** 1.25 µg RNA from fetal rat osteoblasts cultured for given lengths of time as previously described [Aronow et al., 1990]. For limb samples, 2.5 µg of total RNA was used. No product resulted from reactions not containing reverse transcriptase. The apparent decrease after 5 days in culture was not reproducible.

In situ hybridization

Probes used for in situ hybridization were cRNAs of the PCR product described above. After PCR, the product was cloned using the TA cloning kit (Invitrogen). The insert was then transferred into the *Eco*RI site of pBluescript II SK+ phagemid, linearized with either *Bam*HI or *Hind*III, and transcribed at either the T7 or T3 promoter to generate antisense and sense ^{35}S-UTP probes. The sequence of the plasmid insert was identical to the expected region of the published sequence (Fig. 1). Cryostat sections were cut and processed according to Shughrue et al. [1996]. Procedures involving animals were reviewed and approved by the Radnor Animal Care and Use Committee at Wyeth-Ayerst Research.

RESULTS

Figure 2 shows the tissue distribution of meltrin-α in rat tissues when assessed by RT-PCR. All tissues examined contained the message. Similar results were seen with adult mouse skeletal muscle, lung, pituitary, uterus, primary mouse calvaria osteoblasts, MC3T3 cells, and juvenile rat uterus (data not shown). These results indicate that expression of meltrin-α mRNA, when assessed by RT-PCR, is more widespread than was reported using Northern blot analysis. Since our primers share little if any homology with known sequences and the PCR product is identical with meltrin-α, these RT-PCR results reflect meltrin-α gene expression, and not a closely related member of the ADAM family.

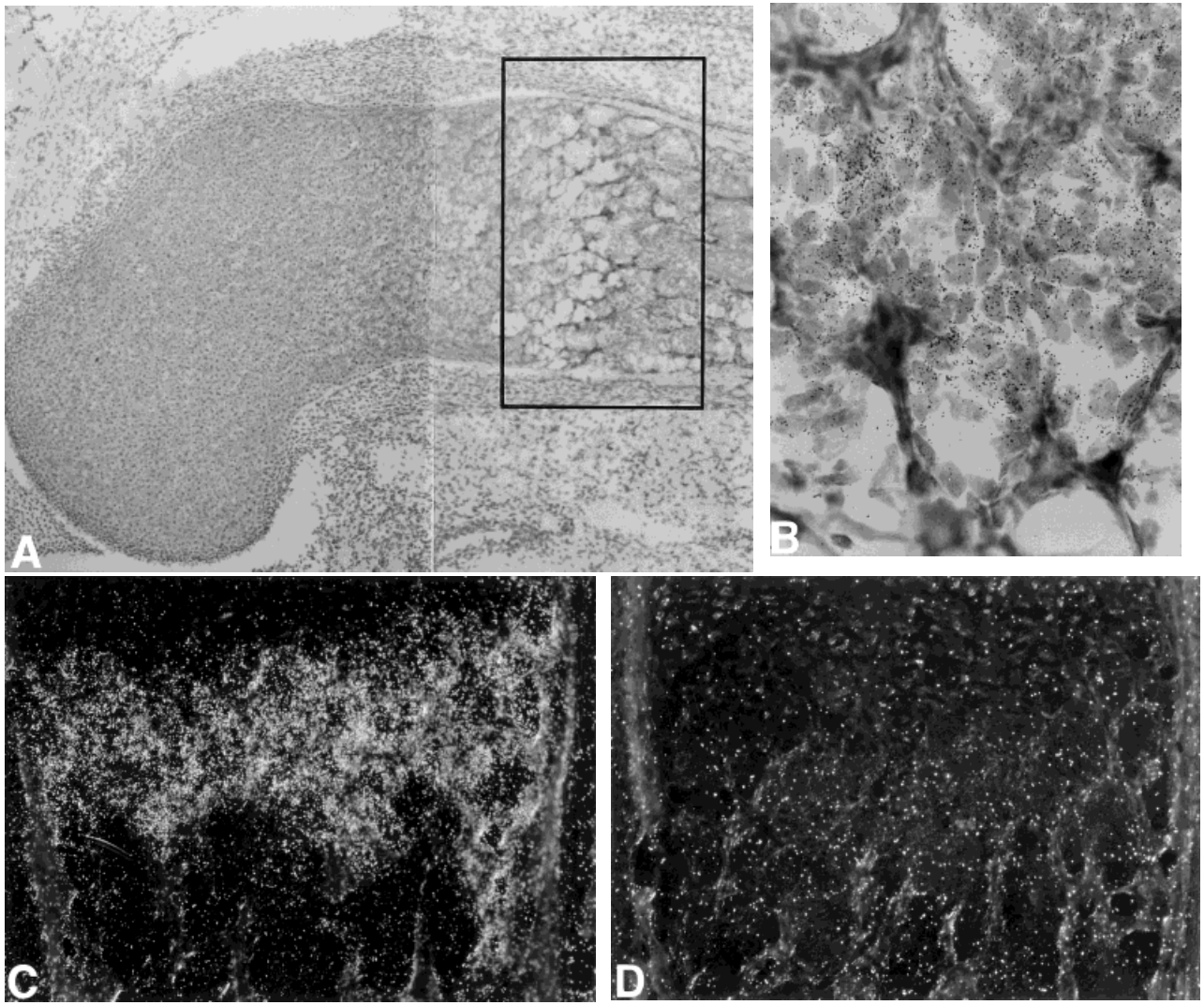

Fig. 4. In situ hybridization showing meltrin-α localization. **A:** Bright-field photo of a sagittal section through a femur hybridized with antisense probe. ×100. **B:** Bright-field view of area boxed in A. ×630. **C:** Dark-field view of the area boxed in A. ×200. **D:** An adjacent section to C hybridized with sense probe. ×200.

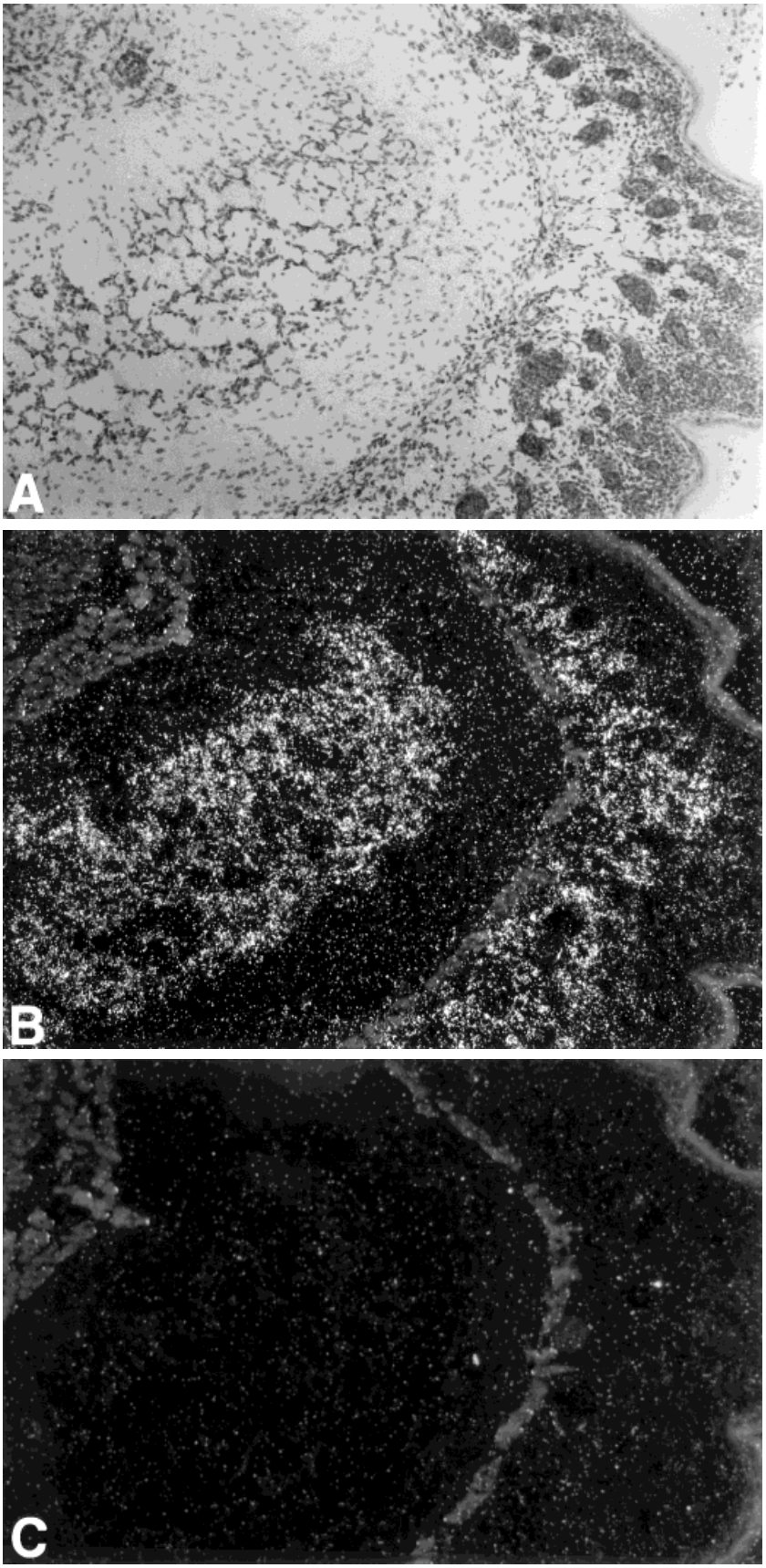

Fig. 5. In situ hybridization showing meltrin-α localization. **A:** Bright-field photo of a sagittal section through the hind limb region showing the skin area hybridized with antisense probe. $\times$100. **B:** Dark-field view of A. **C:** An adjacent section to A hybridized with sense probe.

Given the expression observed in MC3T3 cells, and since our primers readily amplified meltrin-α from rat tissue, we examined expression in cultured rat osteoblasts at different stages of differentiation [Aronow et al., 1990]. As shown in Figure 3, rat osteoblasts express meltrin-α at all stages examined.

The particular abundance in both primary osteoblasts and osteoblastic cell lines is inconsistent with the hypothesis that meltrin-α is expressed only by fusagenic cells such as the osteoclast. To further examine distribution of meltrin-α in bone, in situ hybridization was performed on frozen sections of newborn mice using a cRNA probe synthesized from the PCR fragment shown in Figure 1. As shown in Figure 4, meltrin-α message is located primarily in the trabecular bone subjacent to the growth plate. Because osteoclasts are not abundant in this region, this distribution is consistent with our RT-PCR data indicating a high level of expression by osteoblasts. The periosteum also labeled in some regions (data not shown). Outside of bone, the other area of prominent labeling was the basal region of the dermis and underlying patches of mesenchyme (Fig. 5).

DISCUSSION

The recent publication [Yagami-Hiromasa et al., 1995] identifying myoblast transcripts with homologies to guinea pig fertilin-α and β generated considerable excitement [Miller, 1995] because it suggested a common mechanism between sperm–egg fusion and myotube formation. The evidence that meltrin-α is involved in fusion is suggestive, relying on time of expression and antisense suppression studies. But, as remarked by Miller [1995], a definitive role for meltrin-α in fusion has not yet been proven. As in sperm–egg interaction, other processes (such as adhesion) precede the myoblast fusion event. In addition, the long intracellular tail of meltrin-α may point to a cell signaling role.

The RT-PCR results indicate meltrin-α is widely expressed, suggesting functions other than only mediating myoblast or osteoclast fusion. Moreover, the in situ hybridization studies in neonatal bone show expression of meltrin-α in greater abundance and distribution than would be seen if expression was restricted to the osteoclast. In fact, the distribution pattern agrees with the RT-PCR data showing a high level of expression in the osteoblast. Perhaps meltrin, through its disintegrin domain, mediates adhesion or communication between the osteoblast and other cell types like the osteoclast. The significance of meltrin-α message in the dermis or associated mesenchyme is unknown.

The results presented here do not support the observations and conclusions presented by Yagami-Hiromasa et al. [1995] and Mocharia et al. [1996]. One possible explanation for finding meltrin-α message in a wide variety of tissues is that RT-PCR is a much more sensitive method for studying gene expression than the Northern blotting used by Yagami-Hiromasa et al. [1995]. Also, these authors speculate osteoclast localization of meltrin-α based on their hypothesis that meltrin-α mediates fusion and thus might be expected to be present in the osteoclast or its precursors. However, osteoclast localization was not directly demonstrated. In fact, the RNA used to show bone expression was prepared from total bone which obviously contains a variety of cell types, including osteoblasts. Meltrin-α may indeed be expressed by the osteoclast, but since there are no osteoclast cell lines available and completely pure populations of osteoclasts are unobtainable, one cannot answer this question directly.

Results from Mocharia et al. [1996] are difficult to evaluate and compare to ours since they are presented in an abstract. Because of the conflicting observations, additional studies are needed to better define the role of this newly discovered gene product in cells, particularly in bone.

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REFERENCES

Aronow MA, Gerstenfeld, LC, Owen TA, Tassinari MS, Stein GS, Lian JB (1990): Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvaria cells. J Cell Physiol 143:213–221.

Blobel CP, Wolfsberg TG, Turck CW, Myles DG, Primakoff P, White JM (1992): A potential fusion peptide and an integrin ligand domain in a protein active in sperm–egg fusion. Nature (Lond.) 356:248–252.

Durrer P, Galli C, Hoenke S, Corti C, Gluck R, Vorherr T, Brunner J (1996): H^+-induced membrane insertion of influenza virus hemagglutinin involves the HA2 amino-terminal fusion peptide but not the coiled coil region. J Biol Chem 271:13417–13421.

Miller J (1995): Making one cell from two. Nature (Lond.) 377:575–576.

Mocharia H, Yamate T, Taguchi T, O'Brien CA, Manolagas SC, Abe E (1996): α-Meltrin, a new protein involved in multinucleated giant cell and osteoclast formation. J Bone Miner Res 11(suppl 1):S140 (abst 184).

Myles DG, Kimmel LH, Blobel CP, White JM, Primakoff P (1994): Identification of a binding site in the disintegrin domain of fertilin required for sperm–egg fusion. Proc Natl Acad Sci USA 91:4195–4198.

Podbilewicz B (1996): ADM-1, a protein with metalloprotease- and disintegrin-like domains is expressed in syncitial organs, sperm and sheath cells of sensory organs in *Caenorhabditis elagans*. Mol Biol Cell 7:1877–1893.

Rodriguez CI, Guitierrez G, Encinar JA, Gonzalez JM, Albar JP, Peterson DL, Gavilanes F (1996): Structural properties of the putative fusion peptide of hepatitis B virus upon interaction with phospholipids: Circular dichroism and Fourier-transform infared spectroscopy studies. Eur J Biochem 242:243–248.

Shughrue PJ, Lane MV, Merchanthaler I (1996): In situ hybridization analysis of the distribution of neurokinin-3 mRNA in the rat central nervous system. J Comp Neurol 372:395–414.

Wolfsberg TG, Bazan JF, Blobel CP, Myles DG, Primakoff P, White JM (1993): The precursor region of a protein active in sperm–egg fusion contains a metalloprotease and a disintegrin domain: Structural, functional, and evolutionary implications. Proc Natl Acad Sci USA 90:10783–10787.

Wolfsberg TG, Primakoff P, Myles DG, White JM (1995): ADAM, a novel family of membrane proteins containing a disintegrin and metalloprotease domain: Multipotential functions in cell–cell and cell–matrix interactions. J Cell Biol 131:275–278.

Yagami-Hiromasa T, Sato T, Kurisaki T, Kamijo K, Nabeshima Y, Fujisawa-Sehara A (1995): A metalloprotease-disintegin participating in myoblast fusion. Nature (Lond) 377:652–656.