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Short Communication

Melanoma Cell Adhesion Molecule (MCAM) expression in the myogenic lineage during early chick embryonic development

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ABSTRACT We describe the expression pattern of cMCAM, a cell adhesion molecule of the immunoglobulin superfamily, in early chick embryonic development by *in situ* hybridisation. An initial ectodermal domain of expression is subsequently expanded, and cMCAM is expressed in the neural crest cells, otic vesicle, heart primordium, notochord and endoderm. In addition, cMCAM expression localises in the myotome once the somite cells have been specified. An *in vitro* murine cellular system allowed us to confirm that MCAM expression coincides with the onset of myogenic cell determination.

KEY WORDS: *MCAM, adhesion, myogenic determination*

MCAM (Melanoma Cell Adhesion Molecule) is a cell adhesion molecule belonging to the Ig-superfamily that is expressed by hemopoietic progenitor cells in embryonic bone marrow, thymocytes, and capillary endothelial cells in late chicken embryogenesis (Vainio *et al.,* 1996). It is identical to avian gicerin which is transiently expressed in the developing Central Nervous System (retina and cerebellum), kidney and in adult heart and gizzard (Taira *et al.,* 1994). MCAM is highly expressed in muscle cells in the adult organism (Vainio *et al.,* 1996). Its human orthologue is MUC18, a marker of tumor progression in human melanoma (Lehmann *et al.,* 1989). MCAM / gicerin is a transmembrane protein with an extracellular domain carrying five Ig-like motifs. Two splice variants have been observed (Alais *et al.,* 2001), encoding transmembrane proteins that vary in their cytoplasmic tail. The long cytoplasmic form of gicerin is involved in extension of microvilli in fibroblastic cells (Okumura *et al.,* 2001).

To date, all published data about MCAM expression profile relies mainly on studies performed in adult tissues by Northern blot, RT-PCR or immunostaining. In this paper we studied the expression profile of *cMCAM* during early chick development by the means of *in situ* hybridisation. We used an RNA probe partially overlapping the extracellular domain of the molecule, therefore able to detect both *cMCAM* isoforms. We found that *cMCAM* is expressed in the non-neural ectoderm, neural crest cells, otic vesicle, heart primordia, notochord, somites and endoderm. In order to characterise the *MCAM* expression in the acquisition of the myogenic phenotype, we used an *in vitro* cellular system.

Expression of cMCAM During Chick Embryonic Development and Myogenic Determination

cMCAM expression was first detected at the end of gastrulation (head fold stage, HH6) in the non-neural ectoderm (Fig. 1A) being more visible in the periphery of the embryo at the border with the neural plate (Fig. 1A, see stars). Tranverse sections at that level stained with antibodies anti-MCAM show that cMCAM expression is excluded from the mesodermic tissue (Fig. 1B). At the level of the neural tissue in the head region at 2 somites stage (HH7) there is a transitory *cMCAM* expression in the dorsal neural folds (Fig. 1C). The cMCAM expression is concise to the dorsal part of the embryo since transverse sections at that level show no staining in the unsegmented paraxial mesoderm (Fig. 1D). *cMCAM* expression starts to be visible in the epithelium of the otic vesicle at 18 somites stage (HH13) (Fig. 1E). Later on, the expression of *cMCAM* expands and by the 28-30 somites stage (HH16) in addition to the non-neural ectoderm *cMCAM* is clearly expressed in the dorsal part of the otic vesicle (Fig. 1 F,G), endoderm, heart, neural crest cells as well as in the somites (Fig. 1G).

In order to provide a precise expression pattern at HH17 stage, we performed serial transverse sections along the AP axis of embryos. The expression in the epidermis persists throughout development (Fig. 2). *cMCAM* is expressed quite strongly but temporally in the neural epithelium of the forebrain region (Fig. 2A). It is highly expressed in the neural crest cells and myotome (Fig. 2 B,C,D,E), and weakly in the roof plate of the neural tube (Fig. 2 C,D). The roof plate expression is restricted at the very anterior trunkal level of the embryo and it might depend on the maturation stage of the roof plate cells. It is strongly expressed in the heart (Fig. 2C) from early stages of myocard primordia. It is expressed in the neural crest cells either exiting the neural tube or already in their migratory pathway (Fig. 2 B,C,D,E). At the posterior level expression is observed in the forming mesonephros (Fig. 2E) and in the notochord, this expression being

Abbreviations used in this paper: AP, antero-posterior; DV dorso-ventral; Ig, immunoglobulin; MCAM, melanoma cell adhesion molecule.

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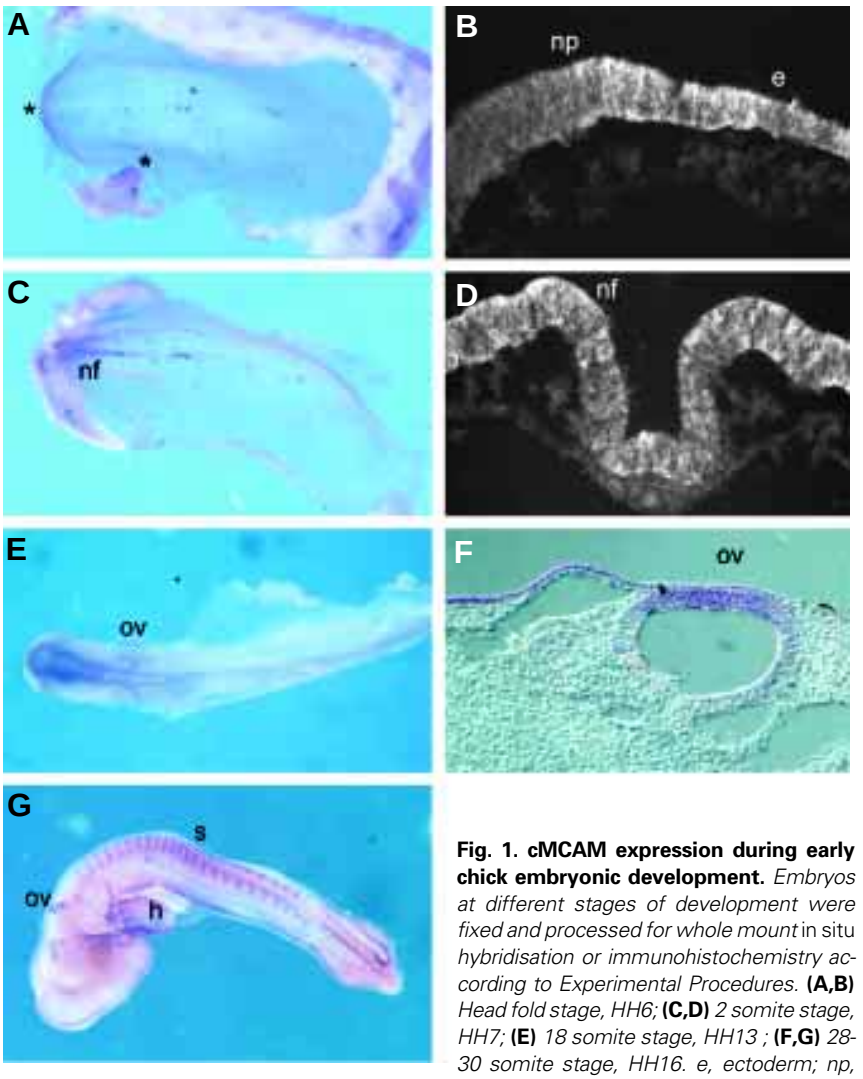

Fig. 1. cMCAM expression during early chick embryonic development. *Embryos at different stages of development were fixed and processed for whole mount* in situ *hybridisation or immunohistochemistry according to Experimental Procedures.* **(A,B)** *Head fold stage, HH6;* **(C,D)** *2 somite stage, HH7;* **(E)** *18 somite stage, HH13 ;* **(F,G)** *28-30 somite stage, HH16. e, ectoderm; np, neural plate; nf, neural folds; ov, otic vesicle; s, somites; h, heart. Anterior is to the left in A, C,E,G, and dorsal is to the top in B,D,F. Stars (*) indicate the periphery of the neural plate.*

highly dynamic along the AP axis (Fig. 3). Somites are formed and mature following a rostrocaudal gradient on either side of the axial structures. Therefore during somitogenesis in the same embryo there is a difference in somite maturation along the AP axis. The anterior trunkal paraxial mesoderm will be already segmented and specified meanwhile the posterior one will still be presomitic paraxial mesoderm. Sections at different trunkal levels of the embryo where somitogenesis is taking place, indicate that expression of *cMCAM* in somites is restricted to the myotome (Fig. 2 B,C,D,E).

Skeletal muscles of the vertebrate body are derived from somites, mesodermal units that segment progressively the paraxial mesoderm in a rostrocaudal succession for an extended period of embryogenesis (Christ and Ordhal, 1994). The somite differentiates along the DV axis to give a dorsal epithelial dermomyotome (muscle and dermis) and a ventral mesenchymal sclerotome (axial skeleton and ribs) (reviewed by Tajbakhsh and Cossu, 1997).

Somite differentiation can be followed by the expression of myogenic markers as *cMyf5* (Tajbakhsh and Cossu, 1997). As previously described, we observed strong *cMyf5* expression in myogenic cells from E2.0 to E.5 (Fig. 3 A-C, and data not shown). From stage 10 onward, *cMyf5* is detected in the dorsal epithelial somite (Fig. 3A) and then in the myotome (Fig. 3 B,C). It has been recently reported a differential expression of *Myf5* dependent on the maturation stage of the somites (Hirsinger *et al.*, 2001). In our study we have observed a strong *cMCAM* expression in the myotome from E2.5 to E4.0 (Fig. 3 D,E, and data not shown). We observed that when somites were not dorsally specified into dermomyogenic progenitors (either due to early embryonic stage or their very caudal position in the AP axis) *cMCAM* expression was not detected in the epithelial somite or in the presomitic paraxial mesoderm (Fig. 3D). However, when somites assess their DV specification (dermomyotome and sclerotome) *cMCAM* becomes to be strongly expressed in the dorsal part of the somite, the myotome (Fig. 3E), and this expression persists onwards. *cMCAM* expression overlays *cFgf8* expression in the myotome (data not shown), a marker of early muscle tissue.

In summary, these data show that meanwhile cMCAM is expressed throughout development in different epithelial tissues like ectoderm, mesonephros and otic vesicle as well as in muscle tissues,

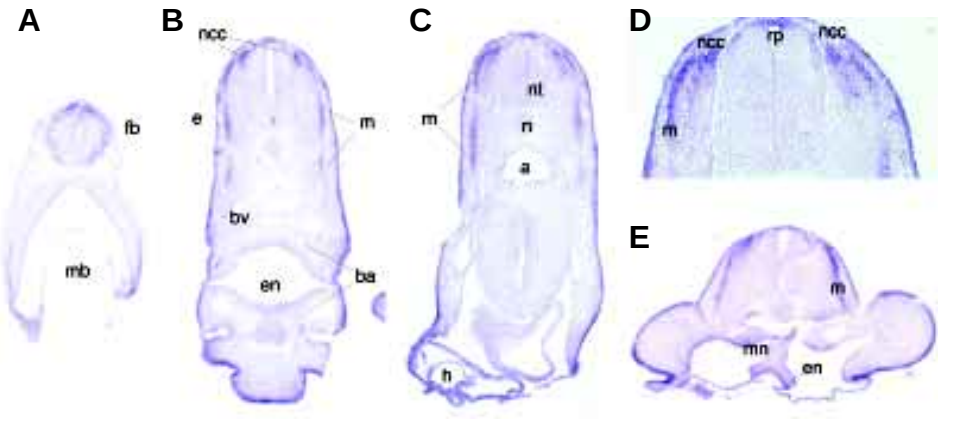

Fig. 2. Expression of cMCAM transcripts along the AP axis at HH17. *Transverse sections at different levels of the AP axis of whole mount* in situ *hybridised embryos at stage HH17.* **(A)** *Anterior head region,* **(B)** *anterior trunkal region,* **(C)** *heart level,* **(D)** *magnification of C,* **(E)** *forelimb level. fb, forebrain; mb, midbrain; ncc, neural crest cells; m, myotome; bv, blood vessel; ba, branchial arch; e, epidermis; en, endoderm; a, aorta; n, notochord; h, heart; nt, neural tube; mn, mesonephros.*

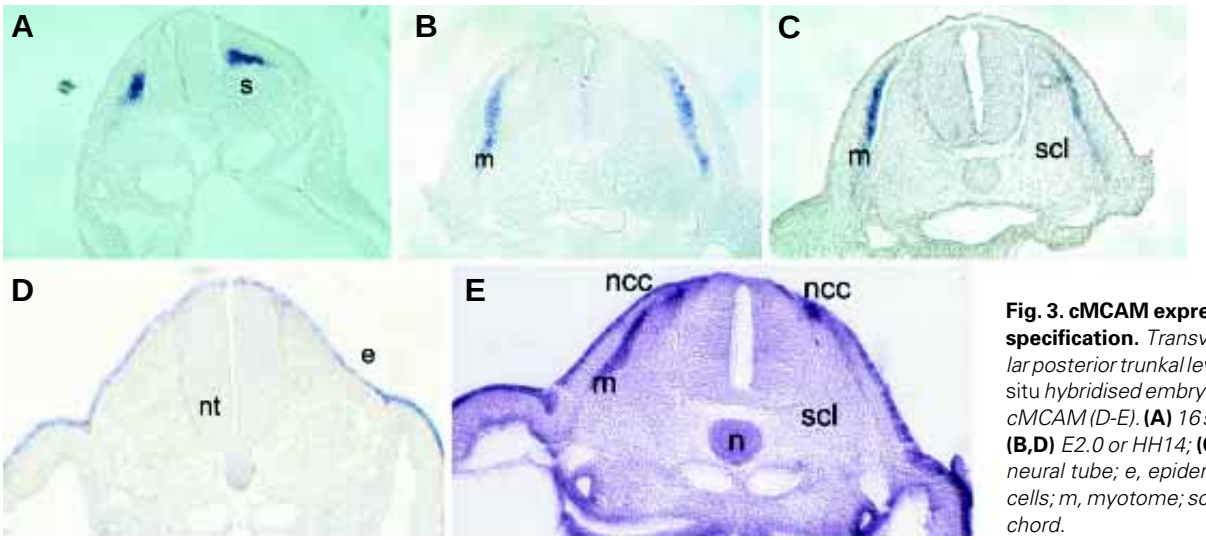

Fig. 3. cMCAM expression during somite specification. *Transverse sections at similar posterior trunkal levels of whole mount* in situ *hybridised embryos with* cMyf5 *(A-C) or cMCAM (D-E).* **(A)** *16 somite stage or HH12;* **(B,D)** *E2.0 or HH14;* **(C,E)** *E3.5 or HH17. nt, neural tube; e, epidermis; ncc, neural crest cells; m, myotome; scl, sclerotome; n, notochord.*

it is only temporary detected in other cells types including neural tissue. After initial specific expression in the non-neural ectoderm, *cMCAM* expression profile broadens and overlays the myotome region once the somite has specified. Our results show *cMCAM* could be used as a new marker of myogenic committed cells.

Expression of MCAM in Culture Occurs upon Induction of Mesodermal Cells to Committed Myogenic Cells

Several laboratories have shown the importance of the MDF (*muscle determination factors*) such as *Myf5* and *MyoD* in promoting myogenesis and have suggested that in mammals and birds axial structures activate myogenesis through a Myf5 dependent pathway while dorsal ectoderm acts through a MyoD dependent pathway. Subsequently, the great majority of myogenic cells express both *MyoD* and *Myf5*, although at variable level. The available evidence suggests that activation of *Myf5* and *MyoD* constitutes the determinative step that commits cells to the myogenic lineage (Tajbakhsh and Cossu, 1997). Recently it has been reported that the differential expression of *Myf5* and *MyoD* depends on the maturation state of the somites (Hirsinger *et al.,* 2001).

To confirm the onset of expression of *MCAM* in the somites with myoblast determination we took advantage of established mouse embryonic fibroblasts, the mesoderm-like 10T1/2 cell line. This cell line is particularly interesting because clonal analyses demonstrated that 5-Azacytidine converts 10T1/2 cells into three stably determined, but undifferentiated, stem cell lineages which can differentiate into myofibers, chondrocytes, and adipocytes (Konieczny and Emerson, 1984). Conversion of 10T1/2 cells by 5-Azacytidine provides us a model for studying myogenic determination, or commitment to cell fate.

Using the RT-PCR procedure, we analysed the expression of *mMCAM* and *Myf5* in cultures of proliferating 10T1/2 cells. As shown in Fig. 4, neither *Myf5* (confirming the mesodermal origin of 10T1/2 cells) nor any of the isoforms of *mMCAM* could be detected. As experimental controls the housekeeping gene *HPRT*, and *SC-1* (Tanaka *et al.,* 1991), another molecule from the same Ig-subgroup than MCAM were used. Upon treatment of 10T1/2 cells with 5-Azacytidine, cells displayed a different gene expression pattern. They did express *Myf5* confirming their conversion into muscle cells (Fig. 4) as previously reported (Montarras *et al.,* 1991), and moreover they started expressing both isoforms of *mMCAM* (Fig. 4). It would be interesting to confirm that the cell population undergoing myogenic determination is the very same one that expresses *mMCAM* since 30% of 10T1/2 cell population undergoes myogenic conversion upon treatment (Konieczny and Emerson, 1984). However, to date there are no available antibodies anti-mMCAM and anti-Myf5 which work in immunofluorescence studies that would allow us to perform this experiment. Nevertheless, no detection of *mMCAM* has been reported or observed neither in chondrocytes nor in adipocytes (the other two stem cell lineages obtained after treatment of 10T1/2 cells), strongly suggesting that indeed the same cell population committed to the myogenic fate expresses *mMCAM*.

These data show that cells do not express *MCAM* if they are not committed to the myogenic pathway and support our *in situ* hybridisation results showing that cells from the paraxial mesoderm do not express *MCAM* unless they are restricted to the dermomyotome. These results clearly support our hypothesis that *MCAM* can be used as a marker for myogenic committed cells, and its expression starts only after cell determination.

Experimental Procedures

In Situ *Hybridisation*

A 514-bp cDNA fragment spanning nt 425 to 939 of the coding region belonging to the extracellular domain of *cMCAM* (HEMCAM in databases) was used to prepare RNA probes. Chicken eggs were

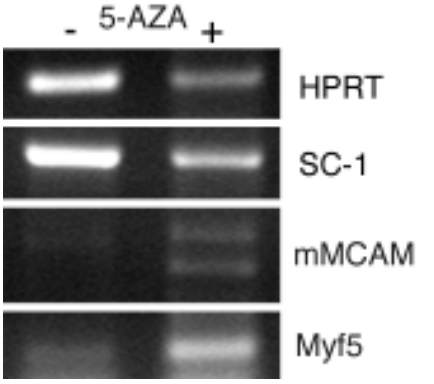

Fig. 4. Expression of MCAM in myogenic derivatives of 10T1/2 cells. *RT-PCR of 10T1/2 cells before (-) and after (+) 5-Azacytidine (5-AZA) treatment for 24h at 37°C. The results were obtained after 25 cycles of PCR amplification. The same results were obtained when PCR reactions were done using 30 cycles of amplification (data not shown).*

incubated at 37°C and staged according to number of somite pairs and staging series of Hamburger and Hamilton. The embryos were fixed overnight with 4% paraformaldehyde in PBS. Whole mount *in situ* hybridisations were performed as described (Wilkinson and Nieto, 1993). For sectioning of whole mounts, embryos were embedded in paraffin and 10µm sections were prepared as described (Pujades *et al.,* 1992).

Immunofluorescence Staining

Embryos were sectioned using a cryostat after fixation with 4% paraformaldehyde/PBS and inclusion in 0.5% Sucrose/PBS. Immunolabeling of cryosections with antibodies against cMCAM was then conducted as described (Alais *et al.,* 2001).

RNA Preparation and RT-PCR

Total RNA preparation, RT-PCR and oligonucleotides for amplification of *Myf5* cDNA were as described (Montarras *et al.,* 1991). Oligonucleotides for amplification of *mMCAM* and *HPRT* cDNA were as published (Alais *et al.,* 2001). For the amplification of the 620bp product of *SC-1* cDNA, oligonucleotides used were:
5' CGA CTG TGG TGT GGA TGA AGG ATA AC,
5' CCA TAT TAC CAA GGT CCT TGT TTA C.

Cell Culture

The embryonic mouse cell line 10T1/2 was obtained from C. Pinset and D. Montarras (Institut Pasteur, Paris). The myogenic derivatives of 10T1/2 cells were obtained by 5-Azacytidine treatment for 24h at 37°C (Konieczny and Emerson, 1984). All cell cultures were performed in DMEM supplemented with 10%FCS in the presence of antibiotics.

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