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Original Article

The homeobox gene *Msx* in development and transdifferentiation of jellyfish striated muscle

SABINA GALLE, NATHALIE YANZE and KATJA SEIPEL*

Institute of Zoology, Biocenter / Pharmacenter, Basel, Switzerland

ABSTRACT Bilaterian *Msx* homeobox genes are generally expressed in areas of cell proliferation and in association with multipotent progenitor cells. Likewise, jellyfish *Msx* is expressed in progenitor cells of the developing entocodon, a cell layer giving rise to the striated and smooth muscles of the medusa. However, in contrast to the bilaterian homologs, *Msx* gene expression is maintained at high levels in the differentiated striated muscle of the medusa *in vivo* and *in vitro*. This tissue exhibits reprogramming competence. Upon induction, the *Msx* gene is immediately switched off in the isolated striated muscle undergoing transdifferentiation, to be upregulated again in the emerging smooth muscle cells which, in a stem cell like manner, undergo quantal cell divisions producing two cell types, a proliferating smooth muscle cell and a differentiating nerve cell. This study indicates that the Msx protein may be a key component of the reprogramming machinery responsible for the extraordinary transdifferentation and regeneration potential of striated muscle in the hydrozoan jellyfish.

KEY WORDS: *jellyfish, muscle specific homeo box, Msx, regeneration, transdifferentiation*

Introduction

The setting aside of undifferentiated embryonic cells is an integral part of development in most animals. The set aside cells are intended for use as somatic stem cells in differentiation and restitution of the adult body and in the germ line. The set aside cells of sponges, some cnidarians and turbellarians are pluripotent or totipotent, able to give rise to a great variety, or possibly all, cell types of the organism. Mammalian adult stem cells, on the other side, appear to have a rather limited potential, usually restricted to one cell lineage. Some urodele amphibians and hydrozoan jellyfish use additional cellular sources for regeneration. They recruit differentiated cells to generate stem cells for regeneration. In this process differentiated cells dedifferentiate, resume cell cycle activity and transdifferentiate to new cell types (Okada, 1991; Eguchi and Kodama, 1993).

One class of regulatory factors specifically associated with regenerative processes is the *Msx* homeo box family (Schummer *et al.,* 1992; Akimenko *et al.,* 1995; Reginelli *et al.,* 1995; Koshiba *et al.,* 1998; Echeverri and Tanaka, 2002). *Msx* genes are found in animals ranging from sponge to mammal (Fjose *et al.,* 1985; Robert *et al.,* 1989; Holland, 1991; Schummer *et al.,* 1992; Seimiya *et al.,* 1994; Aniello, 1999; Gauchat *et al.,* 2000). They have been named for their muscle specific expression and are characterized by a distinct and highly conserved homeo domain of a non-hox type. The sponge homolog Prox3 and human Msx homeo domains are 77% identical (Seimiya *et al.,* 1994). While invertebrates and primitive chordates have single *Msx*-like genes, amphibia and vertebrates feature several *Msx* genes. *Msx* genes are generally expressed in areas of cell proliferation (Davidson, 1995) and in association with multipotent progenitor cells (Hu *et al.,* 2001; Nose *et al.,* 1998).

Msx gene expression has been studied in a variety of regenerative processes. The urodele amphibian limb regeneration is accompanied by expression of both urodele *Msx* genes (Simon, 1995; Echeverri and Tanaka, 2002). While *Msx2* is expressed during early regenerative stages when cells dedifferentiate and a blastema is formed, *Msx1* is upregulated during proliferation of the blastema at the medium to late stages (Koshiba *et al.,* 1998). In fetal and newborn mice *Msx* genes are also expressed in digit tip regeneration, but not during wound healing (Reginelli *et al.,* 1995). Furthermore ectopic expression of mammalian *Msx1* leads to dedifferentiation of mammalian myotubes (Odelberg *et al.,* 2000). In zebrafish expression of the four *Msx* genes is reactivated during fin regeneration (Akimenko *et al.,* 1995). Thus the ability to upregulate *Msx* gene expression may be a prerequisite to regeneration in vertebrates.

Abbreviations used in this paper: Msx, muscle specific homeobox; Cniwi, cnidarian Piwi homolog.

***Address correspondence to:** Dr. Katja Seipel. Institute of Zoology, Biocenter / Pharmacenter, Klingelbergstrasse 50, CH-4056 Basel, Switzerland. Fax: +41-61-267-1627, e-mail: Katja.Seipel@unibas.ch

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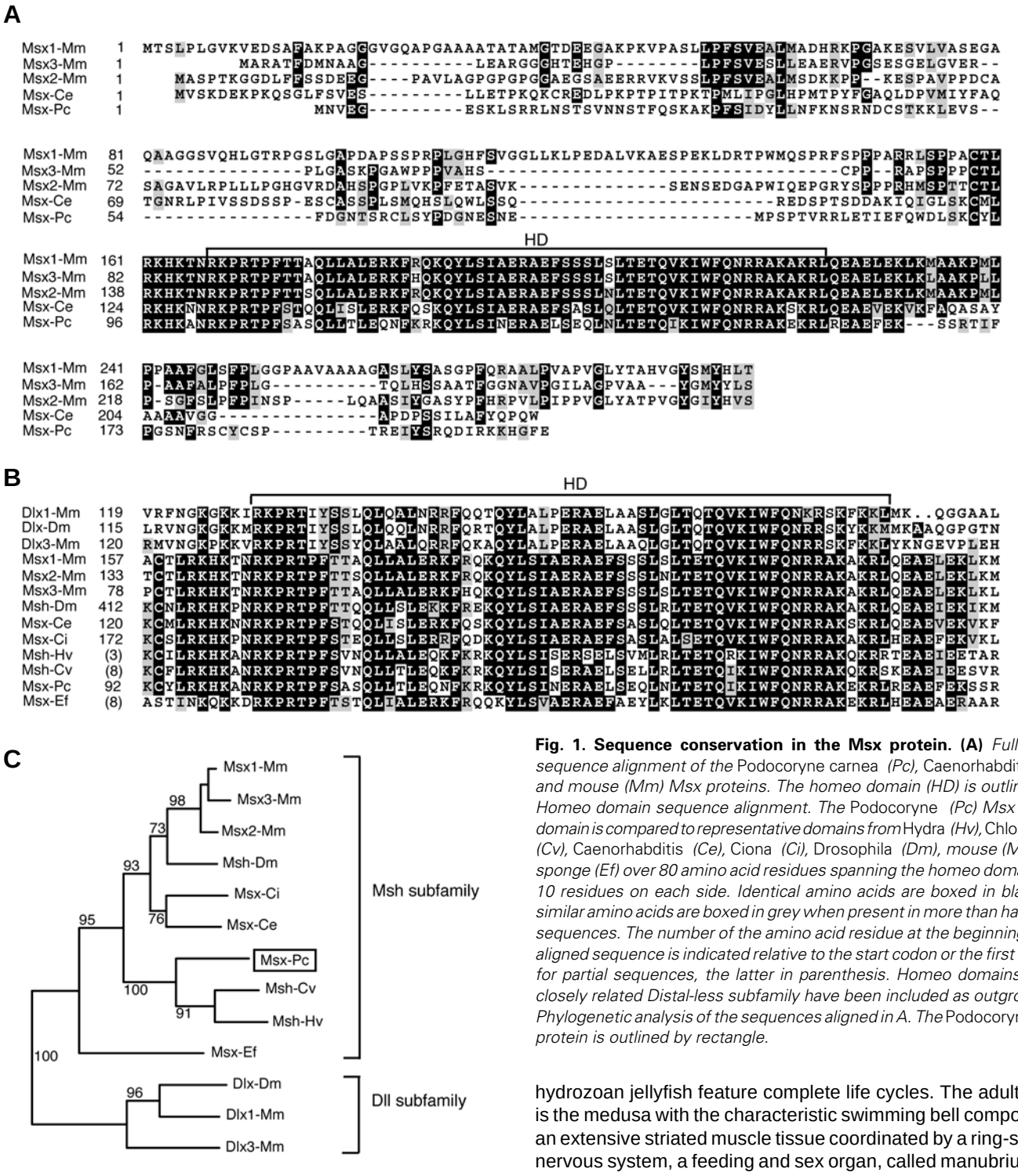

Fig. 1. Sequence conservation in the Msx protein. (A) *Full length sequence alignment of the* Podocoryne carnea *(Pc),* Caenorhabditis *(Ce) and mouse (Mm) Msx proteins. The homeo domain (HD) is outlined.* **(B)** *Homeo domain sequence alignment. The* Podocoryne *(Pc) Msx homeo domain is compared to representative domains from* Hydra *(Hv),* Chlorohydra *(Cv),* Caenorhabditis *(Ce),* Ciona *(Ci),* Drosophila *(Dm), mouse (Mm) and sponge (Ef) over 80 amino acid residues spanning the homeo domain plus 10 residues on each side. Identical amino acids are boxed in black and similar amino acids are boxed in grey when present in more than half of the sequences. The number of the amino acid residue at the beginning of the aligned sequence is indicated relative to the start codon or the first residue for partial sequences, the latter in parenthesis. Homeo domains of the closely related Distal-less subfamily have been included as outgroup.* **(C)** *Phylogenetic analysis of the sequences aligned in A. The* Podocoryne *Msx protein is outlined by rectangle.*

The regeneration potential is extensive in all classes of Cnidaria (Galliot and Schmid, 1992). Regenerative processes have been widely studied in the fresh water polyps *Chlorohydra viridissima (Cv)* and *Hydra vulgaris (Hv).* While the *Cv-Msh* gene is differentially expressed during polyp regeneration (Schummer *et al.,* 1992), the *Hv-Msh* gene is expressed in nerve cells located in the ectodermal layer of the central part of the body column (Miljkovic-Licina *et al.,* 2004). Unlike the fresh water polyps the hydrozoan jellyfish feature complete life cycles. The adult stage is the medusa with the characteristic swimming bell composed of an extensive striated muscle tissue coordinated by a ring-shaped nervous system, a feeding and sex organ, called manubrium and a gastrovascular system. Despite the higher level of tissue organization the hydrozoan medusa exhibits superb regenerative abilities based mainly on the recruitment of differentiated cells by transdifferentiation. In *Phyalidium hemisphaericum* (syn. *Campanularia johnstoni*) interradial umbrellar fragments apparently devoid of undifferentiated cells can regenerate a whole jellyfish (Schmid and Tardent, 1971). In *Podocoryne carnea* (syn. *Hydractinia carnea*) isolation and activation of fully differentiated

striated muscle cells results in transdifferentiation into several distinct cell types, such as smooth muscle cells and RFamide-positive nerve cells, with the potential to form tentacles and manubria *in vitro* (Schmid and Alder, 1984; Alder and Schmid, 1987; Schmid and Reber-Müller, 1995). Here we investigate expression of the jellyfish *Msx* gene in development and transdifferentiation of the striated muscle.

Results

The *Podocoryne carnea Msx* gene was cloned by homology PCR. Analysis of the 776 bp cDNA revealed a 657 bp open reading frame flanked by the start and stop codons at position 51 and 654, respectively. The gene product of 201 residues is closely related in size and sequence to the murine Msx3 protein (Fig. 1A). The most conserved region is the Msx type homeo domain (HD) plus the flanking amino acids. The *Podocoryne* Msx homeo domain is most similar to the *Chlorohydra viridissima* and *Hydra vulgaris* Msh proteins with 87% and 81% amino acid identity, respectively (Fig. 1B), while it shares 74-79% identical residues with the bilaterian and 67% with the sponge Msx proteins. Regions outside the homeo domain are diverse in sequence and size. Nevertheless there is a short motif PFSIDYLL at position 29 in the *Podocoryne* Msx, similar to PFSVESLL at position 27 in the murine Msx3 and PFSVEALM at positions 54 and 47 in the murine Msx1 and Msx2 proteins, respectively. The phylogenetic analysis of the Msx homeo domains confirms the placement of the *Podocoryne* Msx protein within the Msx subfamily of homeo box proteins (Fig. 1C).

Msx transcripts are present in all life cycle stages of the hydrozoan jellyfish *Podocoryne carnea* with highest levels in the egg and in the developing and adult medusa (Fig. 2A and B). The RNA is present in the oocyte as maternal message and with progressively reduced levels in the planula larva and gastrozoid polyp (Fig. 2B). During medusa development RNA levels are low in the early bud stages, increasing to highest levels in bud stages 5 to 6 (Fig. 2C). In excised fragments of the freshly detached medusa *Msx* is expressed at high levels in the striated muscle lining the subumbrellar plate of the medusa bell (Fig. 2D and E). Additionally *Msx* is expressed at very low levels in the other bell tissues and in the tentacles but not in the manubrium (Fig. 2E).

To define the expression pattern in more detail *Msx* transcripts were localized by *in situ* hybridization in the developing medusa. *Msx*- specific staining is evident in the entocodon (Fig. 3A to C) and the entocodon-derived striated muscle (Fig. 3D to I). The entocodon, a mesoderm-like layer originating from the distal ectoderm of the growing bud (reviewed in Seipel and Schmid, 2005) consists of proliferative progenitor cells giving rise to the smooth and striated muscles as well as the RFamide positive nerve cells of the bell (Seipel *et al.,* 2004a). All the cells of the early entocodon appear to be *Msx*-positives (Fig. 3A). At stages 3 to 4, when the entocodon forms a cavity, the future subumbrellar space, *Msx* -specific staining is progressively restricted to the lateral entocodon (Fig. 3B and C) which consists of muscle progenitor cells that have initiated myofilament assembly (Boelsterli, 1977). There is no detectable staining in the distal entocodon (asterisk in Fig. 3B and C) or in the smooth muscle of the manubrium (arrow in Fig. 3D and G). Moreover *Msx* -specific staining appears to be restricted to the interradial regions of the striated muscle layer (Fig. 3F) in direct contact to the interradial endoderm plate. There is no staining in the differentiating striated muscle tissue over the radial canals (arrowhead in Fig. 3F). In the late stages *Msx* expression is diminished throughout the striated muscle (Fig. 3H and I). The margin of the striated muscle constituting the inner layer of the velum is clearly visible in the whole mount staining as it is folded back onto the striated muscle of the

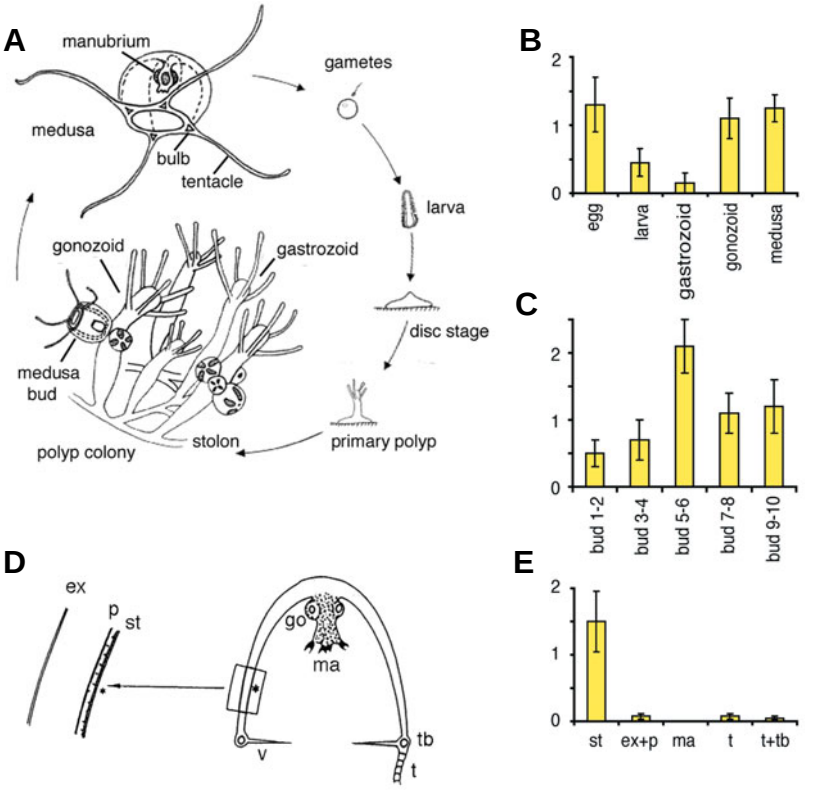

Fig. 2. *Msx* expression in the jellyfish life cycle. (A) *Life cycle. The fertilized egg of* Podocoryne carnea *develops into a ciliated planula larva within 30 hours. It attaches to a suitable substrate and transforms into the primary polyp. After stolon outgrowth at the base of the primary polyp a colony of feeding polyps (gastrozoids) and medusa budding polyps (gonozoids) develop asexually. In the mid-body region of the gonozoid, medusa buds of different developmental stages form a whorl. The medusa buds develop into medusae in 7 to 8 days and are classified according to their stage of differentiation from 1 to 10 (Frey, 1968).* **(B)** *Quantitative RT-PCR expression analysis of the* Msx *gene in different life cycle stages.* **(C)** *Quantitative RT-PCR expression analysis of the* Msx *gene in different bud stages of medusa development.* **(D)** *Cross-section through the adult medusa with magnified view of the medusa bell. Asterisk indicates striated muscle layer.* **(E)** *Quantitative RT-PCR expression analysis in different excised parts of the freshly detached medusa. The ordinate values are relative expression levels normalized to the expression level of* EF1α *and displayed in arbitrary units. Abbreviations: ex, exumbrella; go, gonads; ma, manubrium; p, plate endoderm; st, striated muscle; tb, tentacle bulb; t, tentacle; v, velum.*

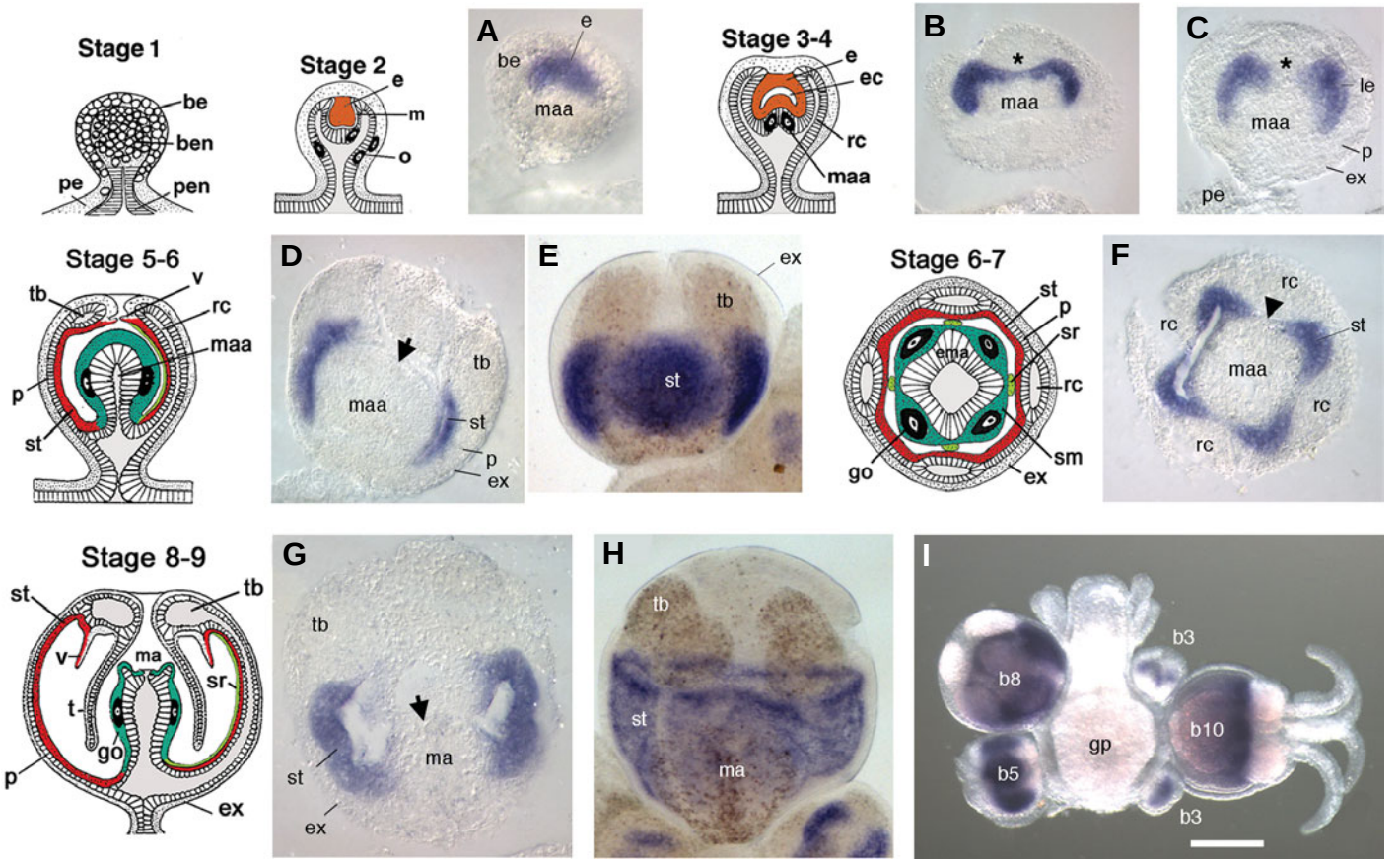

Fig. 3. *Msx* expression in the developing medusa. In situ *hybridization with* Msx *probe in medusa bud stages 2-3* **(A)**, *3-4* **(B,C)**, *6* **(D-F)**, *8* **(G)**, *9* **(H)** *and in gonozoid polyp* **(I)** *with medusa buds of stages 3, 5, 8 and 10. (A-D) sagittal sections; (F) cross section, left side slightly frayed and cracked; (G) oblique sagittal section; (E, H, I) whole mount, lateral view.* Msx- *specific staining localizes to the distal entocodon in stages 3-4 and the entocodon derived striated muscle in stages 5-10. Asterisk indicates distal entocodon in (B,C). Arrow points to smooth muscle of the manubrium in (D,G). Arrowhead points to striated muscle over the radial canal in (F).* Msx *signal intensity is maximal in the developing striated muscle of bud stage 5. At bud stage 10 the tentacles have unfolded and the medusa is ready to detach from the gonozoid polyp.* Msx- *specific staining is absent from the gonozoid polyp. Bar corresponds to 100 μm in (A to F), 200 μm in (G, H) and 400 μm in (I). Abbreviations: b3, b5, b8, b10, bud stages 3-10; be, bud ectoderm; ben, bud endoderm; ema, endoderm of the manubrium; ex, exumbrella; go, gonads; gp, gonozoid polyp; ma, manubrium; maa, manubrium anlage; p, plate endoderm; pe, polyp ectoderm; pen, polyp endoderm; rc, radial canal; sm, smooth muscle of the manubrium; sr, smooth muscle over the radial canal; st, striated muscle; tb, tentacle bulb; t, tentacle; v, velum.*

bell thereby doubling the staining intensity (Fig. 3H). In contrast to the *Hydra* polyp (Schummer *et al.,* 1992; Miljkovic-Licina *et al.,* 2004), the *Podocoryne carnea* gastrozoid and gonozoid polyps appear to have little or no *Msx* expression as there is specific staining in the medusa buds but not in the polyp itself (Fig. 3I). In summary, the developmental expression territory of the jellyfish *Msx* gene comprising the entire entocodon in the early medusa bud is progressively confined to the developing striated muscle and then to the differentiated striated muscle in the interradial segments of the medusa bell, thus including both progenitors as well as differentiated striated muscle cells.

In order to analyze *Msx* gene expression during transdifferentiation the striated muscle tissue was mechanically isolated from the interradial regions of the medusa bell and cultured in sea water. In the absence of further treatments (Fig. 4A), the striated muscle cells stably maintain the differentiated state as well as the expression levels of striated muscle specific genes (Schmid *et al.,* 1998; Yanze *et al.,* 1999). Likewise *Msx* transcript levels are continuously maintained at high levels in the striated muscle tissue (Fig. 4B). However, when isolated with collagenase and treated with pronase the striated muscle cells change the differentiation state, start to proliferate and divide asymmetrically producing smooth muscle and sensory nerve cells (Fig. 4C). Within one hour after pronase treatment *Msx* expression is turned off and the RNA level is strongly reduced in the dedifferentiating striated muscle cells (Fig. 4D). In this initial phase the stem cell gene *Cniwi* is shortly activated and silenced again (Seipel *et al.,* 2004b). In the two days following these very early events the *Msx* gene is silent and the RNA levels are very low (Fig. 4D). During this time the cells acquire a smooth muscle phenotype and re-enter cell cycle (Alder and Schmid, 1987) while *Cniwi* gene expression is upregulated again reaching maximal transcript levels on day 3 (Seipel *et al.,* 2004b) when *Msx* expression is strongly activated (Fig. 4D). At the same time the *Atonal* gene is activated (Seipel *et al.,* 2004a). The first asymmetric cell division produces a proliferating stem cell and a nerve cell.

The expression level of the *Polycomb* gene rises in conjunction with cell differentiation (Lichtneckert *et al.,* 2002). After day 4, the proliferation rate slows down and the *Msx* transcript level gradually decreases (Fig. 4D) consistent with a silencing of *Msx* expression in the differentiating nerve cells which start to express the neuronal marker gene *NP* coding for the RFamide neuropeptide (Seipel *et al.,* 2004a).

In summary the jellyfish *Msx* gene is strongly expressed 1) in the entocodal progenitor cells giving rise to striated muscle 2) in the quiescent differentiated striated muscle with transdifferention capability and 3) in the stem cells arising by transdifferentiation from the striated muscle cells.

Discussion

Jellyfish are the most basal metazoan organisms with clearly defined striated muscle layers based on ultrastructural and molecular studies. The jellyfish striated muscle derives from the entocodon, a third cell layer established at the onset of medusa development by separation from the ectoderm. This raised the possibility of entocodon-mesoderm equivalence which was substantiated by the discovery of conserved mesoderm and myogenic regulators in the jellyfish (reviewed in Seipel and Schmid, 2005). The jellyfish *Msx* gene is yet another exponent corroborating the common origin hypothesis as gene expression is most prominent in the entocodon and derived striated muscle of the developing medusa and in the quiescent differentiated striated muscle of the adult medusa. Muscle specificity has first been described in *Drosophila* (Fjose *et al.,* 1985) where *Msx* is expressed in the muscle progenitors of the somatic mesoderm (Nose *et al.,* 1998). In vertebrate development *Msx* gene expression is associated with multipotent progenitor cells in a wide range of tissues (Bendall and Abate-Shen, 2000). The vertebrate *Msx* genes apparently function to maintain *Cyclin D1* expression in progenitor populations during development, thereby preventing exit from cell cycle and terminal differentiation (Hu *et al.,* 2001). The jellyfish *Msx* gene is expressed in the muscle progenitors of the entocodon (Fig. 3B), the differentiated striated muscle (Fig. 4B) and the neuronal progenitors arising by transdifferentiation from the striated muscle (Fig. 4D). Although the striated muscle cells do not proliferate, all of these cell types are progenitor cells, in the sense that they represent intermediate stages to certain differentiation products. It is possible that other progenitor cells also express *Msx*, as there is low level expression in most, but not all, tissues (Fig. 2E).

Msx gene expression in the striated muscle is positively regulated by specific cell-ECM interactions. Disruption of these cell-ECM interactions leads to immediate gene silencing and RNA degradation of the *Msx* (Fig. 4D), as well as of diverse muscle specific structural and regulatory genes (Yanze *et al.,* 1999). Reactivation of the *Msx* gene likely depends on the re-establishment of specific cell-ECM interactions. As *Msx*-specific staining is restricted to the interradial regions of the striated muscle (Fig. 3D), there are also several negative gene regulatory pathways. The radial regions of the striated muscle are characterized by *Brachyury* gene expression (Spring *et al.,* 2002). It thus appears that striated muscle expression of the *Msx* and *Brachyury* genes is mutually exclusive in the interradial and radial regions, respectively, opening the possibility of a reciprocal inhibition of gene expression in the distinct muscle expression territories. Moreover, the interradial regions are characterized by *Twist* gene expression in the plate endoderm (Spring *et al.,* 2000), in direct contact to the *Msx* expressing striated muscle, while the radial regions are devoid of both *Twist* and *Msx* gene transcripts. This expression pattern is consistent with the presumptive inhibitory function of the Twist protein on muscle specific genes (Spring *et al.,* 2000), in this case the inhibition of *Msx* gene expression in the plate endoderm.

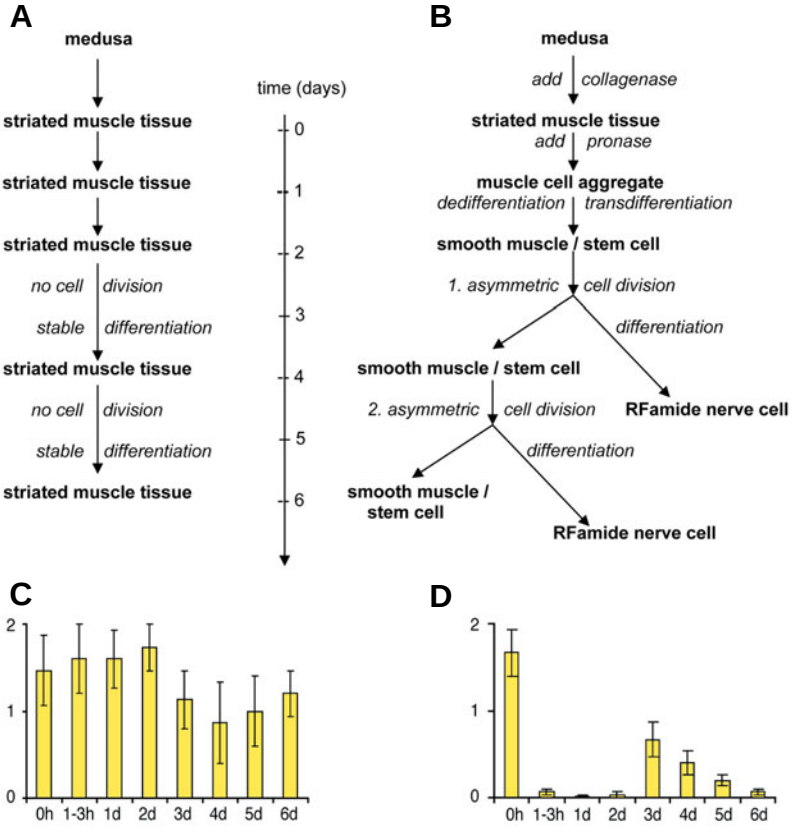

Fig. 4. *Msx* expression during *in vitro* transdifferentiation. **(A)** *Flow chart of the control experiment. The striated muscle tissue and adhering ECM is isolated mechanically and cultured on glass in sea water. Without further treatment the striated muscle stably maintains its differentiated state for several days.* **(B)** *Quantitative RT-PCR expression analysis of the* Msx *gene in the isolated striated muscle (see A).* **(C)** *Flow chart of the transdifferentiation experiment. The striated muscle is isolated with collagenase followed by pronase treatment to initiate transdifferentiation. The striated muscle cells transdifferentiate to smooth muscle cells which start to divide asymmetrically producing proliferating smooth muscle cells and precursors of RFamide neuropeptide positive sensory nerve cells.* **(D)** *Quantitative RT-PCR expression analysis of the* Msx *gene in the activated striated muscle (see C) and emerging cell types. The ordinate values are relative expression levels normalized to the expression level of* EF1α *and displayed in arbitrary units.*

The primary signal for transdifferentiation in the jellyfish muscle, according to various destabilization experiments, arises by disturbance of cell-ECM interactions and the signaling involves membrane receptors connected to the cytoskeleton as well as PKC activation (Schmid and Reber-Müller, 1995). The activating treatment leads to rapid upregulation of the stem cell gene *Cniwi* (Seipel *et al.,* 2004b) and to silencing of the *Msx* gene with complete degradation of the *Msx* transcripts within the hour (Fig. 4D). As Piwi proteins are implied in gene silencing and RNA interference processes (Tahbaz *et al.,* 2004), the jellyfish Cniwi protein may be involved in *Msx* gene silencing and/or RNA degradation. It remains to be determined whether *Msx* gene silencing is functionally related to transdifferentiation and/or S-phase re-entry.

In summary, *Msx* appears to be involved in progenitor cell maintenance, both in proliferating progenitor cells during development and regeneration and in quiescent differentiated cells with transdifferentiation capability.

Materials and Methods

Animals

Podocoryne carnea M. Sars (Cnidaria, Hydrozoa, Anthomedusae) colonies are reared in the laboratory in aerated artificial seawater at 16°C. Animals were cultured on glass jars and fed every second day with two days old artemia. Medusa buds were staged according to Frey (1968). Eggs and larval stages were obtained as described (Seipel *et al.,* 2004).

Isolation of striated muscle tissue and initiation of transdifferentiation

For control experiments striated muscle tissue consisting of mononucleated cells and a thin layer of adhering ECM was isolated mechanically by microsurgery from jellyfish freshly liberated from the gonozoid polyps. The isolated tissue fragments were cultured on glass in sterile artificial seawater at 22°C. For transdifferentiation experiments striated muscle tissue was isolated from jellyfish incubated at room temperature in 150 units/ml collagenase (Type III, fraction A, Sigma) for several hours to digest the inner mesoglea thus separating the striated muscle layer from the subumbrellar plate (Schmid and Alder, 1984). To achieve 100% initation of transdifferentiation the isolated muscle was incubated in 1.25mg/ml pronase (*Streptomyces griseus* pronase, Boehringer) for 2-3 min. at room temperature (Schmid *et al.,* 1993).

Molecular cloning and sequence comparison

Molecular biology procedures have been carried out as described (Seipel *et al.,* 2004a). mRNA used for homology PCR was extracted from *Podocoryne carnea* medusa using the Dynabeads Direct kit (Dynal). First strand cDNA synthesis was primed with the oligonucleotide XT20V (5' - GGC AGG TCC TCG TTG CGA GAC GT (20) (AGC) - 3') using the First Strand cDNA Synthesis kit (Roche). Homology PCR for *Msx* was performed using the primers MSXA (5'- GAA CAA AA(AG) TT(CT) AAA (AC)G(ACGT) AA(AG) CA - 3') and HOXF (5'- ACA AGC TTG AAT TCA T(ACGT)C (GT)(AG)T T(CT)T G(AG)A ACC - 3'). Homology PCR was performed under standard conditions with an annealing temperature of 40°C for 40 cycles. The fragment of the expected size was gel purified with a Qiaquick column (Quiagen), subcloned into the pCRII-TOPO vector (TOPO TA cloning Dual promotor kit, Invitrogen) and sequenced on ABI PRISM 310 genetic analyser (PE Applied Biosystems) with BigDye in combination with the halfTERM Reagent (GENPAK). 3' RACE was performed on the directed λZAPII cDNA library made from *Podocoryne carnea* gonozoids and medusa (Müller *et al.,* 1999) with MSF1 (5'- ACC TAT CGA TAA ATG AAC GTG CCG - 3') and T7XL (5'- GAA TTG TAA TAC GAC TCA CTA TAG GGC G - 3'), MSR1 (5'- CGG CAC GTT CAT TTA TCG ATA GG - 3') and T3XL (5'- GCT CGA AAT TAA CCC TCA CTA AAG GG -3'). 5'RACE was performed on cDNA using the SMART RACE cDNA Amplification kit (Clontech). The resulting fragments were amplified with Expand High Fidelity PCR System, purified with Qiaquick column, subcloned into the pCRII-TOPO vector and sequenced. The *Msx* coding sequence has been submitted to the DNA databases with accession number AY874426. Nucleotide and deduced amino acid sequences were analyzed using the GCG software package. BLAST searches (Altschul *et al.,* 1997) were performed using the BLAST network service at the NCBI (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments and phylogenetic trees based on the neighbour-joining method were generated with Clustal X (Jeanmougin *et al.,* 1998). Accession numbers for published protein sequences used in Fig. 1 are as follows: Dlx-Dm, AAB24059; Dlx1-Mm, AAB40899; Dlx3-Mm, AAB38415; Msh-Cv, CAA45912; Msh-Dm, AAC47329; Msh-Hv, CAB88390; Msx-Ce, NP_509648; Msx-Ci, CAB42631; Msx-Ef, S43227; Msx1-Mm, AAH16426; Msx2-Mm, Q03358; Msx3-Mm, NP_034966.

Relative quantification of gene expression by RT-PCR

mRNA from eggs, larvae, polyps, medusae and excised medusa parts was extracted using the Dynabeads mRNA Direct Kit (Dynal) as recommended by the manufacturer. 1st strand cDNA synthesis and real time PCR were carried out as described (Seipel *et al.,* 2004a). The ubiquitously expressed elongation factor 1 alpha (EF1α) was included in each set of experiments as a reference to compensate for variations in the quantity and quality of preparations. PCR analysis was done in duplicates of at least three independent experiments. *Msx* expression was evaluated using the primers MSF3 (5'- CGCGAA GAT TGA ACT CGA CTT C -3') and MSR1 (5'- CGG ACG TTC ATT TAT CGA TAG G -3'). Primers for *EF1α* have been described (Yanze *et al.,* 1999).

In situ *hybridization*

In situ hybridization was carried out as described previously (Seipel *et al.,* 2004a). Detection was performed by immunochemical staining with anti-DIG-Fab-AP (Roche) using NBT/BCIP as a substrate. Specimens were fixed for 2 hours at room temperature with freshly prepared 4% paraformaldehyde or Lavdowsky solution (ethanol: 37% formaldehyde: glacial acetic acid: water = 50: 10: 4: 36). Linearized plasmid pCRII-TOPO (TOPO TA cloning, Invitrogen) containing the *Msx* homeo box was used as template to generate digoxigenin labelled antisense probes by *in vitro* runoff transcription with the DIG-RNA Labelling kit (Roche) and T7 RNA polymerase (Roche). Hybridization was carried out at 64°C for 16h. Detection was performed by immunostaining with anti-DIG-Fab-AP (Roche) using NBT/BCIP as a substrate. For sectioning stained specimens were postfixed for 2 hours at room temperature in 4% paraformaldehyde, dehydrated in ethanol, embedded in Paraplast (Sigma) and sectioned to 10μm.

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