Int. J. Dev. Biol. 50: 499-503 (2006)
doi: 10.1387/ijdb.052115dk

Short Communication

Msx1 and Msx2 have shared essential functions in neural crest but may be dispensable in epidermis and axis formation in *Xenopus*

DEEPAK KHADKA#, TING LUO and THOMAS D. SARGENT*

Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda MD, USA

ABSTRACT The homeodomain factors Msx1 and Msx2 are expressed in essentially identical patterns in the epidermis and neural crest of *Xenopus* embryos during neurula stages. Disruption of *Msx1* and *Msx2* RNA splicing with antisense morpholino oligonucleotides shows that both factors are also required for expression of the neural crest gene *Slug*. Loss of Msx1 can be compensated by overexpression of Msx2 and vice versa. Loss of Msx factors also leads to alterations in the expression boundaries for neural and epidermal genes, but does not prevent or reduce expression of epidermal keratin in ventrolateral ectoderm, nor is there a detectable effect on dorsal mesodermal marker gene expression. These results indicate that Msx1 and Msx2 are both essential for neural crest development, but that the two genes have the same function in this tissue. If *Msx* genes have important functions in epidermis or axial mesoderm induction, these functions must be shared with other regulatory proteins.

KEY WORDS: *homeodomain factor, morpholino antisense oligonucleotide*

The Msx homeodomain transcription factors are vertebrate homologs of the muscle-segment homeobox (msh) gene in Drosophila melanogaster. There are two or three Msx genes in human and mouse respectively(Bendall and Abate-Shen, 2000; Davidson, 1995), five in zebrafish (Ekker *et al.,* 1997) and two have been described in *Xenopus*, Msx1 and Msx2, originally named Xhox7.1 and Xhox7.1' (Su *et al.,* 1991). Msx factors function generally as repressors, potentially acting through multiple mechanisms, including interference with members of the Dlx homeodomain factor family(Zhang *et al.,* 1996; Zhang *et al.,* 1997), which are transcriptional activators, interaction with histone H1b (Lee *et al.,* 2004) and via interactions with a conserved engrailed homology region in the amino terminal portion of the protein (Smith and Jaynes, 1996). Loss of Msx gene function by mutation or targeted inactivation results in defects in craniofacial skeleton, tooth and limb development (Alappat *et al.,* 2003). Combined inactivation of Msx1 and Msx2 yields a considerably stronger phenotype in both cranial and cardiac NC (Ishii *et al.,* 2005). In *Xenopus*, Msx1 has been implicated in dorsoventral axis specification (Maeda *et al.,* 1997; Takeda *et al.,* 2000), epidermal development (Suzuki *et al.,* 1997) and has recently been identified as an early regulatory factor in the induction of neural crest (Monsoro-Burq *et al.,* 2005; Tribulo *et al.,* 2003; Tribulo *et al.,* 2004). In order to distinguish between possibly different functions of Msx1 and Msx2 and to provide a means to remove Msx gene products without resorting to dominant negative strategies, we have developed antisense morpholino oligonucleotides (MOs) that efficiently and specifically block splicing of Msx1 and Msx2 RNAs in the *Xenopus* embryo. Using these MOs we confirm the importance of Msx1 in neural crest induction, show that Msx2 plays an equivalent role and that both Msx1 and Msx2 are necessary for NC. In contrast, loss of Msx expression does not affect axial specification or epidermal development, suggesting that these functions are either redundant with other factors or alternatively, were incorrectly attributed to Msx based on misinterpretation of overexpression experiments.

The *Xenopus* homologs of mammalian Msx1 and Msx2 were initially named Xhox7.1 and Xhox7.1' respectively (Su *et al.,* 1991; GenBank Accession X58773 and X58772). These two

Abbreviations used in this paper: MO, morpholino oligonucleotide; msh, muscle segment homeobox.

***Address correspondence to:** Dr. Thomas D. Sargent. Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892, USA. Fax: +1-301-496-0243. email: tsargent@nih.gov

#Current address: Department of Biochemistry, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA.

0214-6282/2006/$25.00

Printed in Spain
www.intjdevbiol.com

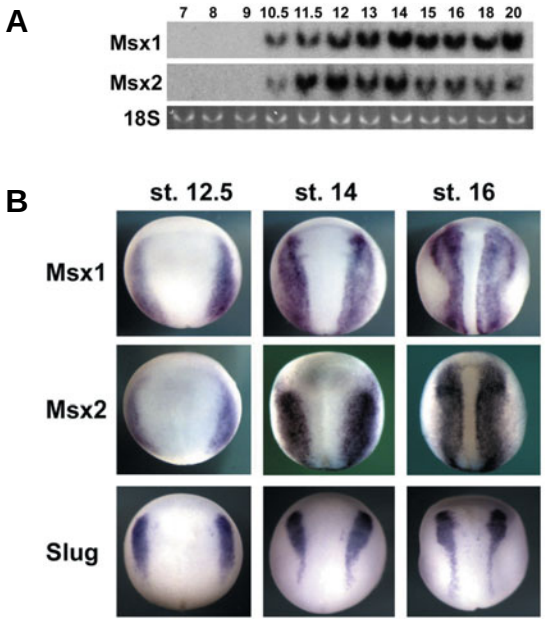

Fig. 1. Expression of *Msx1* and *Msx2* in *Xenopus* embryos. (A) *Glyoxal RNA gel blots of 0.75 μg per lane of total RNA isolated from different embryonic stages probed with* Msx1 *and* Msx2. *Nieuwkoop-Faber stages are as indicated. Both transcripts appear first at early gastrula, level off at early neurula and are maintained at roughly constant amounts through mid tailbud stages. Ethidium bromide staining of the 18S ribosomal band is shown to control for equal RNA loading.* **(B)** *Whole mount* in situ *hybridization to late gastrula (St. 12.5) and early (St. 14) and mid-neurula (St. 16) embryos. The expression patterns of* Msx1 *and* Msx2 *are very similar. Expression of* Slug *is shown for comparison. Dorsal views, with anterior towards the top of the figure.*

proteins factors share 55% overall amino acid identity, with 58/60 identical residues within the homeodomains and thus are genuinely different genes and not due to the pseudotetraploid nature of the *Xenopus laevis* genome. Some expression data have been reported for both genes, but the two have not been directly compared. Figure 1A shows a developmental northern series, revealing that both Msx1 and Msx2 RNAs first appear at the beginning of gastrulation (stage 10.5) and remain at roughly constant and roughly equal, levels through organogenesis (stage 20). Whole mount *in situ* hybridization of Msx1 and Msx2 probes to late gastrula through mid neurula embryos is shown in Fig. 1B. Again, both Msx1 and Msx2 expression patterns are very similar, with strong signals found at the neural-epidermal border, in the region of neural crest induction. As has been reported previously (Tribulo *et al.,* 2003), when compared to the NC marker slug, the Msx genes exhibit a broader expression domain, particularly in the posterior/trunk region. This supports the conclusion that there is a basic difference between the regulation of Msx gene expression and that of NC induction in general (Monsoro-Burq *et al.,* 2005).

To further investigate this point animal cap experiments were carried out in which variable doses of RNA encoding the BMP antagonist chordin were injected with a fixed dose (300 pg) of Wnt3a RNA. This combination has been shown to efficiently induce NC gene expression in such explants (LaBonne and Bronner-Fraser, 1998; Saint-Jeannet *et al.,* 1997), at a specific intermediate level of BMP signaling (Luo *et al.,* 2003; Tribulo *et al.,* 2003). As shown in Fig. 2, both Msx1 and Msx2 are induced by a broad range of chordin doses, compared to Slug and Sox9, another marker for NC and otic placode (Spokony *et al.,* 2002), which require higher chordin levels. In fact, injection of Wnt3a RNA alone induces both Msx genes nearly as much as when it is combined with chordin RNA. Another property of the Msx genes is revealed at the 5 ng chordin RNA dose, which is sufficient to extinguish BMP signaling (Luo *et al.,* 2003) and leads to the silencing of other NC markers. Both Msx1 and Msx2 are only partially inhibited under these conditions, returning to a level approximately equal to that of untreated ectoderm (UI). Since Msx gene expression is strongly inhibited by even low doses of BMP antagonists administered alone (Feledy *et al.,* 1999; Suzuki *et al.,* 1997) this expression also supports the notion that Msx genes can be positively regulated by canonical Wnt signaling, independently of BMP signals.

In order to design morpholino antisense oligonucleotides that interfere with the splicing of the single intron in Msx genes, genomic DNA fragments spanning *Xenopus laevis* Msx1 and Msx2 intron/exon boundaries were amplified by polymerase chain reaction. Primers were derived from the mRNA sequences, based on the prediction that the intron in *Xenopus* Msx genes would be at the positions equivalent to those in mouse and human Msx genes. These DNA fragments were sequenced and used to generate two MOs that spanned the splice sites. The Msx1 MO sequence (M1) was 5'-TATAGGGTCAACTTACTTGTTGGG-3' and was targeted to the splice donor site (first two nucleotides of intron are underlined). The Msx2 MO (M2) sequence was 5'-CACTCTCTTTTTTAGGACACCTGAGCC – 3' and was targeted to the splice acceptor (last two nucleotides of intron underlined). The specificity and effectiveness of these MOs is shown in Fig. 3. Northern blot analysis was performed on RNA from whole embryos collected at early neurula stage (st 14). At this stage Msx expression is primarily ectodermal, but some residual contribution from mesodermal tissue would be included in these assays. A dose of 30 ng per embryo of M1 (M1) eliminated approximately

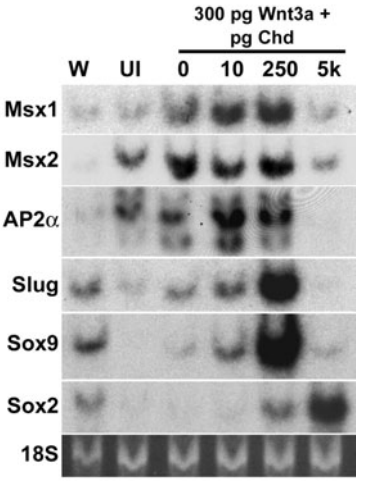

Fig. 2. Animal cap assay showing dependence of *Msx* and other neural and neural plate gene expression on canonical Wnt and modulated BMP signaling. *Ectoderm was isolated from mid/late blastula stage embryos (St. 7-8) injected at the one-cell stage with 300 pg of* Wnt3a *RNA and variable amounts of* chordin *RNA, ranging from zero to 5 ng, cultured to early neurula (St. 14) and processed for Northern blot analysis using methylmercury hydroxide agarose gel electrophoresis. Note that compared to the other NC genes* AP2a, Slug *and* Sox9, Msx *genes are up-regulated significantly by Wnt3a alone and also are not fully extinguished by the highest dose of chordin, which activates neural plate genes such as* Sox2. *UI, uninjected animal caps; W, whole embryo. Ethidium bromide staining of 18S ribosomal RNA is shown to control for equal RNA loading.*

80% of correctly spliced Msx1 transcripts without affecting Msx2 significantly. Likewise, 10 ng of M2 had the reciprocal effect – elimination of about 80% of Msx2 RNA with minimal effect on Msx1 transcripts. Note that in both cases there were some weak higher molecular weight bands present, presumably generated by abortive splicing (not shown).

Figure 4 shows the effects of these MOs on gene expression in embryos injected into one cell at the two-cell stage, with the dose adjusted down to 15 ng M1 and 5 ng M2, accordingly. MOs were injected along with 250 pg ,β-galactosidase as a lineage tracer that was detected by staining with X-gal. Knockdown of either Msx1 or Msx2 resulted in very strong inhibition in the expression of neural crest marker Slug (84%, n=68 and 100%, n=62, respectively). As a control for the specificity of this effect and also to determine the degree of functional redundancy of Msx1 and Msx2, rescue experiments were carried out in which blastomeres injected with 15 ng of M1 were co-injected with 10 pg of Msx2 mRNA and blastomeres injected with 5 ng of M2 were co-injected with 5 pg of Msx1 mRNA (there is no significant homology between the two MOs). These mRNA quantities are 50-100X lower compared to the amounts used in similar studies (Feledy *et al.,* 1999; Tribulo *et al.,* 2003; 2004; Monsoro-Burq *et al.,* 2005). In addition to preventing unwanted ventralization effects, these lower doses may also help avoid potential overexpression artifacts: 5 pg/embryo is roughly equivalent to the concentration of a typical rare class endogenous mRNA in the *Xenopus* embryo (Sargent and Dawid, 1983). In both cases substantial rescue was observed: Msx1 mRNA yielded 55% normal expression, 25% weakly repressed and 20% strongly repressed (n=28), while Msx2 mRNA yielded 50% normal expression, 20% weakly repressed and 30% strongly repressed (n=32). Thus both Msx1 and Msx2 are required for NC induction, as measured by Slug expression, but have essentially interchangeable function in this context.

These results data indicate that the role of Msx1/2 is shared and losing either leads to NC induction failure. In the mouse, the single Msx1 knockout has a much weaker phenotype than the double Msx1/Msx2 (Ishii *et al.,* 2005). The simplest interpretation of this is that the threshold for Msx1+2 levels is lower in the mouse than in the frog, such that losing half the total (e.g. single knockout) has a relatively minor effect. Alternatively, mouse Msx genes may be more specialized and less critical for initial induction events, leading to differential knockout phenotypes.

Msx factors have been implicated, based on overexpression and on dominant-negative experiments, in the establishment of dorsal-ventral polarity in *Xenopus* mesoderm (Gong and Kiba, 1999; Maeda *et al.,* 1997; Takeda *et al.,* 2000) and also the activation of epidermal and repression of neural gene expression programs in *Xenopus* ectoderm (Feledy *et al.,* 1999; Suzuki *et al.,* 1997). One potential problem with all of these experiments is that due to redundancy in DNA binding specificity, the potential for heterodimer formation and other potentially confounding properties, overexpression of full-length or dominant-negative constructs of Msx factors may be prone to some nonspecific artifacts. To investigate these functions using the antisense morpholino approach, which does not have these difficulties, embryos were injected into one cell at the two-cell stage with a mixture of 15 ng M1 and 5 ng M2 along with either 2 ng fluorescein-labeled standard control MO (GeneTools) or 250 pg , β-galactosidase as a tracer, intended to maximize the inhibition of Msx protein expression. As shown in Fig. 5A, this resulted in a lateral shift of the epidermal/neural boundary (70%, n=20), but did not prevent expression of epidermal keratin in ventral ectoderm.

A markedly different result was obtained with axial mesoderm markers (Fig. 5B). Expression of chordin, a marker for the notochord (Sasai *et al.,* 1994), was affected only slightly, in most cases showing no expansion on the injected side (79%, n=19). Similar results (100%, n=25)were obtained with Xnot1, another notochord marker gene(von Dassow *et al.,* 1993). To rule out the possibility that this might be due to uneven diffusion of MO following injection, a mixture of M1 and M2 was injected into the ventral marginal zone at the 1-cell stage and embryos were fixed at early gastrula (St. 10.5) for *in situ* hybridization with chordin. Little if any alteration in the chordin expression domain resulted from inhibition of Msx expression (Fig. 5C; 100%, n=20).

These results confirm earlier findings (Monsoro-Burq *et al.,* 2005; Tribulo *et al.,* 2003) with respect to the function of Msx1 in

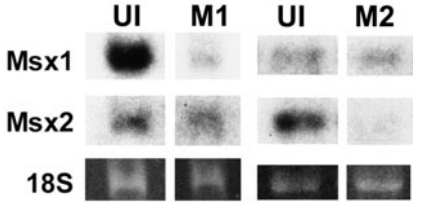

Fig. 3. Effectiveness and specificity of antisense morpholino oligonucleotides (MOs). *Fertilized eggs were injected with 30 ng of* Msx1 (M1) *or* 10 ng Msx2 (M2) *splice-targeted MOs, which were then cultured to early neurula (St. 14) along with uninjected embryos (UI) and processed for Northern blot analysis with* Msx1 *and* Msx2 *probes.* Msx1 *and* Msx2 *transcripts were reduced to a baseline of approximately 20% of control levels, as measured by densitometry of X-ray films, respectively. Neither MO affected the non-homologous RNA, demonstrating specificity. Ethidium bromide staining of 18S ribosomal RNA is shown to control for equal RNA loading.*

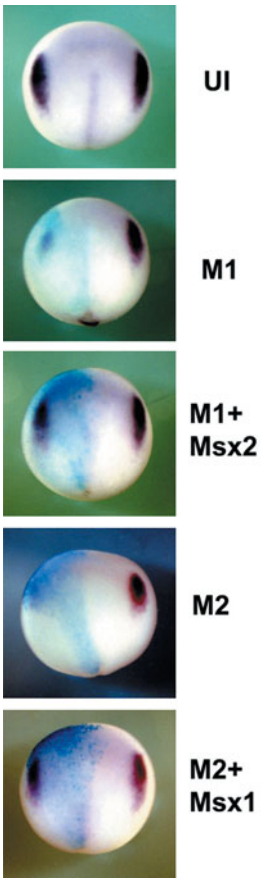

Fig. 4. Loss of Msx function. *Two-cell stage embryos were injected at a single site in one blastomere with 15 ng* M1*, 5 ng* M2*, along with 250 pg* β-galactosidase *RNA as a lineage tracer and cultured to early neurula (St. 13), then fixed, stained with X-gal and processed for whole mount* in situ *hybridization with probes for the neural crest marker gene* Slug*. Dorsal views, with the hybridization signals represented by purple color and the lineage tracer by light blue. The injected side is on the left in all cases and anterior is towards the top of the Figure. Both* M1 *and* M2 *greatly reduced the expression of* Slug*. The* Slug *expression domains were also shifted laterally in some cases. Near-normal levels of* Slug *expression were rescued in a majority of embryos by co-injection of* Msx1 *mRNA (5 pg, with 5 ng* M2*) and* Msx2 *RNA (10 pg, with 15 ng* M1*). An uninjected embryo is shown for comparison (UI).*

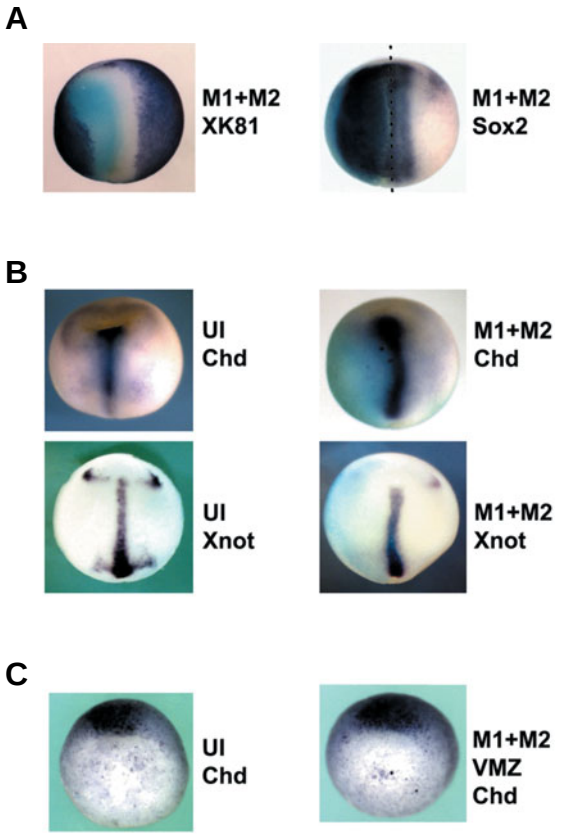

Fig. 5. Effect of combined *Msx1/Msx2* knockdown on the dorsal/ventral axis. *Embryos were injected into one blastomere at the two-cell stage with a mixture of 15 ng* M1 *and 5 ng* M2 *(A,B) or twice this dose at the one-cell stage (C). Fluorescein-labeled standard control MO was used as a tracer in all injections except the one which was used for hybridization to* Xnot*, in which case β-galactosidase was used as the tracer.* **(A)** *Expression at stage 14 of the neural plate marker* Sox2 *was expanded laterally, while the epidermal boundary, indicated by* XK81*, retreated to a corresponding degree. The axial midline is indicated by the dashed vertical line.* **(B)** *Notochord expression at stage 14 of* chordin *and* Xnot1 *were not noticeably affected by* Msx *knockdown. The lateral expression domains of* Xnot1 *were largely eliminated, however. Uninjected controls are shown for comparison (UI).* **(C)** *Embryos injected at the one-cell stage into the ventral marginal zone with 30 ng* M1 *+ 10 ng* M2 *were cultured to stage 10.5, fixed and probed for* chordin *expression. Vegetal views, with dorsal towards the top of the figure. No significant difference was observed between injected and uninjected controls (UI).*

NC development and also reveal this function is shared between Msx1 and Msx2, with full expression of both genes necessary. On the other hand, Msx factors may not be required for epidermal development or for axial specification in mesoderm. This apparent lack of function could be attributed to redundancy with other ventral mesoderm factors such as Vent (Gawantka *et al.,* 1995) or Vox (Schmidt *et al.,* 1996). Another alternative is that the residual Msx RNA escaping the blockage of RNA splicing is sufficient for epidermal and mesodermal functions, but it seems unlikely that a reduction of this magnitude would not have any visible effect. It is interesting in this context to note that insertional inactivation of Msx 1 and Msx 2 genes in mouse do not result in axial disturbances, nor have effects on epidermal specification been reported (Bei *et al.,* 2004). On the other hand it is clear that BMP signal modulation is central to dorsoventral axis formation across animal phylogeny, including *Xenopus* (Dale and Wardle, 1999; Hammerschmidt and Mullins, 2002; Yamamoto and Oelgeschlager, 2004). There is also evidence that Msx1 is a direct target of BMP4 signaling in the frog embryo (Suzuki *et al.,* 1997). The most likely conclusion is that in *Xenopus* Msx1 and Msx2 share an essential function in NC development, particularly concerning the establishment or maintenance of the epidermal/neural boundary, but are less critical, or possibly even dispensable, in epidermis and axial specification.

Experimental Procedures

Embryo Manipulation

Embryos were obtained from adult *Xenopus laevis* by standard hormone-induced egg laying and artificial fertilization procedure (Sive, 1999) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). For ectodermal explant experiments, RNA and morpholino antisense oligonucleotides (MOs) were microinjected into the animal hemisphere at one or two-cell stage. Ectodermal explants were removed at stage 8†and cultured until stage 14, then processed for Northern blot analysis.

Constructs

The open reading frame of *Xenopus* Msx1, starting with the amino acid sequence MAPALLMASYQPGVK, was amplified from a *Xenopus* Hox7.1 (gift of F. Ramirez) and cloned into ClaI and XhoI sites of a modified pCS2+ vector(Feledy *et al.,* 1999). To prepare RNA for injection, plasmid was linearized with Asp718 and transcribed with T7 polymerase. Msx2 RNA was transcribed from IMAGE clone number 5155540 (GenBank Accession CA792675) with SP6 polymerase after linearizing with Not1. Transcription of capped RNA was carried out using a mMessage Machine kit (Ambion, Inc.) according to manufacturer's instructions.

Northern Blot Hybridization

RNAs were isolated and analyzed by using denaturing methylmercury hydroxide RNA gels as described (Sargent *et al.,* 1986) or glyoxal RNA gels (NorthernMax Gly-TM; Ambion Inc.) Radiolabeled DNA probes were prepared by primer extension (Ready-To-Go labeling beads; Amersham Bioscience) with inserts prepared from plasmids for AP2α (Winning *et al.,* 1991), Slug (Mayor *et al.,* 1995), Sox9 (Spokony *et al.,* 2002), Sox2 (Kishi *et al.,* 2000), Msx1 and Msx2 (Su *et al.,* 1991). Staining of 18S ribosomal RNA with ethidium bromide was used to monitor equal loading of RNA samples.

In situ hybridization

Whole-mount *in situ* hybridization was carried out as described (Harland, 1991; Luo *et al.,* 2003). Antisense probes labeled with digoxigenin were synthesized by using a RNA labeling kit (Roche Molecular Biochemicals) with cDNA templates encoding Slug, Sox2, chordin, Xnot1 (von Dassow *et al.,* 1993) and XK81 (Jonas *et al.,* 1985).

Acknowledgements

We thank Dr. Yanhui Zhang for helpful comments and advice on experiments. This research was supported by the Intramural Research Program of the NICHD, NIH.

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Received: October 2005
Reviewed by Referees: November 2005
Modified by Authors and Accepted for Publication: December 2005