IJAE
Vol. 118, n. 1 (Suppl.): 62-65, 2013
ITALIAN JOURNAL OF ANATOMY AND EMBRYOLOGY

# Mechanisms for Relaxin's Modulation of MMPs and Matrix Loss in Fibrocartilages

Sunil Kapila[1], Young Park[1], Nisar Ahmad[1], Jun Hosomichi[1,2], Takayuki Hayami[1], Claire Tacon[3]

[1] Department of Orthodontics and Pediatric Dentistry, the University of Michigan. 1011 North University Avenue, Ann Arbor, Michigan
[2] Department of Orofacial Development and Function, Tokyo Medical and Dental University, Tokyo, Japan
[3] Department of Medicine and Department of Physiology and Pharmacology, University of Calgary, Calgary, Canada

Our previous studies (Hashem et al., 2006; Kapila, 2009; Naqvi et al., 2005) have shown that the fibrocartilaginous temporomandibular joint (TMJ) disc and pubic symphysis respond to relaxin by upregulation of MMPs and loss of key matrix molecules. These responses to relaxin are enhanced by estrogen, and are also modulated by estrogen alone. Since these fibrocartilaginous tissue are heterogeneous containing fibroblastic, chondrocytic and intermediate cell types, the responses of specific cell types to relaxin are not known. Also the direct effect of specific MMPs induced by relaxin to the loss cartilage matrix *in vivo* has not been demonstrated. Our purpose was to (1) characterize cell type-specific response(s) to relaxin and estrogen; (2) identify the relaxin receptor and downstream signaling involved in relaxin's induction of specific MMPs; (3) develop a mouse model for *in vivo* manipulation of hormones; and (4) identify the contribution of each of these hormones and the MMPs they regulate to *in vivo* matrix loss.

Two each of fibroblastic and chondrocytic female mouse TMJ disc cell clones immortalized by human telomerase reverse transcriptase were isolated and characterized on the basis of phenotype, growth curves, and fibroblastic and chondroctytic markers, respectively. Chondrocytic cell clones had higher mRNA and protein expression levels of cartilage oligomeric matrix protein (COMP), collagen X, total collagen, collagen II, and collagen II/collagen I mRNA ratios relative to fibroblastic cell clones. Fibroblastic cell clones had higher mRNA and protein expression levels of vimentin and fibroblastic specific protein 1 (FSP1) than the chondrocytic clones. The fibroblastic cell clones expressed higher levels of relaxin receptors RXFP1 (>6) and RXFP2 (>9) while chondroctyic cell clones had higher level of estrogen receptor, ESR2 (>4.5) compared to their counterparts. Consequently the fibroblastic cells showed greater upregulation of MMP-9 (>2.5) and -13 (>9) and relaxin receptors (>2) by relaxin than the chondrocytic cell clones, while the reverse was true for cellular responses to estrogen.

Next using gene overexpression and suppression strategies we found that RXFP1 but not RXFP2 is involved in the upregulation of MMP-9 and -13 in primary TMJ fibrocartilaginous cells (Fig. 1). Studies using chemical inhibitors and siRNAs to signaling molecules revealed that relaxin induces MMP-9 via PI3K, Akt, ERK and PKC-ζ and the transcription factors Elk-1, c-fos and to a lesser extent NF-κB (Ahmed et al., 2012). MMP-9 promoter-luciferase experiments demonstrated the involvement of the elements responsive to these transcription factors, namely Ets/Pea3, AP-1 and NF-κB, respectively, in relaxin's modulation of MMP-9 (Fig. 2).

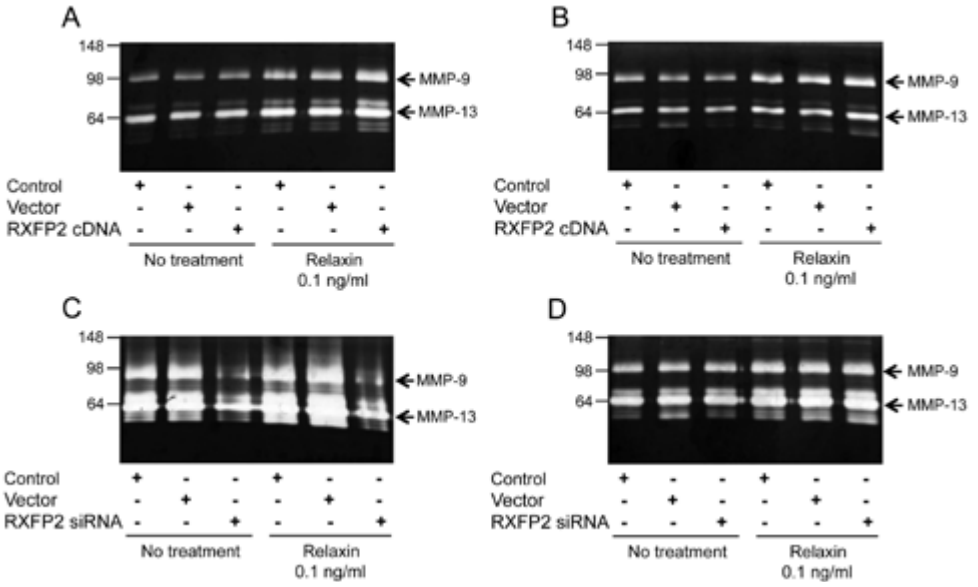


**Figure 1.** RXFP1 but not RXFP2 is involved in relaxin's induction of MMPs in TMJ fibrochondrocytes. A and B: Untransfected cells or cells transiently transfected with pcDNA vector, or RXFP1 or RXFP2 cDNA constructs were cultured in the absence or presence of relaxin. Cell-conditioned medium was collected after 48 hours and assayed by gelatin substrate zymography. Relaxin induced MMP-9 and -13. This response to relaxin was enhanced in RXFP1 but not RXFP2 cDNA transfected cells. C and D: Untransfected cells or cells transiently transfected with scrambled siRNA or siRNA to RXFP1 or RXFP2 were cultured in the absence or presence of relaxin for 48 hours. Cell-conditioned medium assayed by gelatin substrate zymography showed that relaxin's induction of MMP-9 and -13 was inhibited in RXFP1 but not RXFP2 siRNA transfected cells.

We next assessed the contribution of relaxin-induced MMP-9 to matrix loss *in vivo*. One week following bilateral ovariectomies in 12 week-old female C57BL/6 WT or MMP-9 null mice, osmotic pumps containing PBS or relaxin or estrogen or a combination of these hormones were implanted subcutaneously. Six days later, the mice were euthanized, blood was collected and TMJ discs retrieved. The discs were analyzed for collagen, glycosaminoglycan, and MMPs, while the serum was assayed for the hormones. The administered doses of the hormones resulted in systemic hormone concentrations similar to those in cycling women. *In vivo* administration of relaxin and/or estrogen contributed to the upregulation of MMP-9 and -13 concomitant with the loss of matrices from disc in WT mice. Unexpectedly, treatment of MMP-9 null mice with estrogen or relaxin also resulted in loss of disc collagen and glycosaminoglycan to a similar extent as that in WT mice. This finding could be explained by our observation that relaxin and estrogen caused significantly greater induction of MMP-13 in MMP-9 null vs. WT mice, suggesting that MMP-13 may be compensating for the absence of MMP-9 in relaxin-mediated matrix loss.

The findings demonstrate that relaxin and estrogen enhance matrix loss in the TMJ disc concomitant with the induction of their respective receptors, MMP-9 and MMP-13, which are cell-type specific responses. Also the similarities between WT and

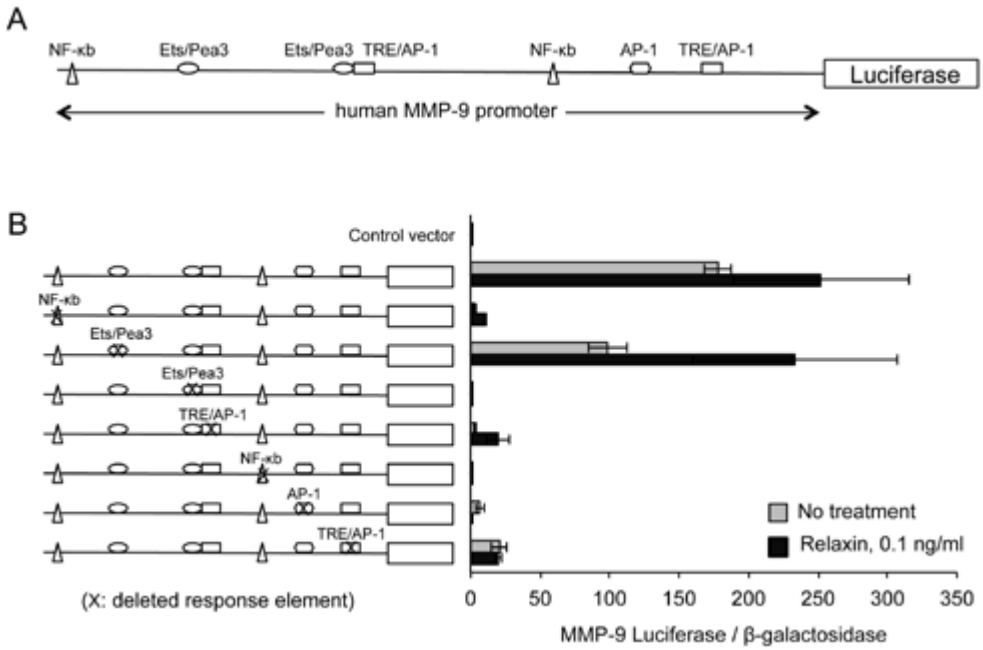


**Figure 2.** The induction of MMP-9 by relaxin in TMJ fibrochondrocytes requires NF-κB, Ets/Pea3 and AP-1 response elements in the human MMP-9 promoter. A: Human MMP-9 promoter luciferase construct utilized showing response key elements. B: Cells transfected with control vector or vector containing wild type or one of seven MMP-9 promoter-luciferase constructs each containing a deleted response element were cultured in the absence or presence of relaxin for 6 hours. Cells were lysed and assayed for luciferase and b-galactosidase; the latter was used for standardizing the luciferase activity. Deletion of NF-κB, Ets/Pea3 and AP-1 response elements diminished the baseline activation as well as relaxin's induction of the human MMP-9 promoter. The results shown represent means (+SE) of three independent experiments.

MMP-9 null mice in the loss of matrix molecules on treatment with estrogen and/or relaxin suggest that both MMP-9 and MMP-13 may act together in contributing to tissue turnover, or mediators other than MMPs may be involved in the enhanced matrix turnover mediated by these hormones.

## Acknowledgment

This work was supported by NIH/NIDCR RO1DE018455.

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