THE EXPRESSION OF MESODERM INDUCTION - EARLY RESPONSE 1 PROTEINS IN EMBRYONIC AND ADULT MOUSE TISSUES

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THE EXPRESSION OF MESODERM INDUCTION - EARLY RESPONSE 1 PROTEINS IN EMBRYONIC AND ADULT MOUSE TISSUES

by

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ABSTRACT

The mesoderm induction early response-1 gene *(mi-er1)* is a fibroblast growth factor-inducible early response gene. It is activated during mesoderm induction in *Xenopus* embryos and it encodes a nuclear protein that functions as a transcriptional regulator. The human orthologue of this gene has 12 distinct transcripts encoding six protein isoforms, which differ in their amino (N) and carboxy (C) terminal domains. The C-terminal variants, alpha and beta, differ in both the size and sequence of their C-terminal domain.

Recently, the mouse orthologue of this gene was cloned and characterized and this study led us to determine the expression pattern of the MI-ER1 protein in mouse embryonic and adult tissues by immunohistochemistry. MI-ER1 was expressed in all mouse tissues analyzed, with an overall decrease in expression in the adult compared to embryonic tissues. MI-ER1 beta was the only isoform expressed in the embryo, while both MI-ER1 alpha and beta were expressed in adult tissues. In the adult, the alpha isoform was particularly expressed at a high level in endocrine tissues, such as the adrenal gland, pancreas, ovary, and testis. There was distinct pattern of MI-ER1 expression in the adult mid-brain, with high expression in the CA3 cells of the hippocampus and the pyramidal cells of the thalamus.

A complex pattern of MI-ER1 alpha and beta subcellular localization was found in the embryo and adult tissues of the mouse. In the embryo, the beta isoform was found in both the nucleus and the cytoplasm. In adult tissues, the beta isoform was found in the nucleus (and possibly in the cytoplasm in some instances), while the alpha isoform was

found in the cytoplasm. The only exception to this was the adult testis, where MI-ER1 alpha was found in both the nucleus and cytoplasm. Also, the spleen was the only adult tissue analyzed where MI-ER1 alpha was not expressed and where we could definitely conclude that MI-ER1 beta was located in the cytoplasm.

The results of the current study were a powerful tool in providing the temporal and spatial expression of MI-ER1 in embryonic and adult mouse tissues. This work will be very significant for future work in the production of transgenic mice to further investigate functions of MI-ER1.

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ABC	Avidin-Biotin-Peroxidase Complex
Akt	protein kinase B
ATP	adenosine triphosphate
BSA	bovine serum albumin
CA3	field CA3 of the hippocampus proper
CBP	CREB binding protein
C/EBP beta	CCAAT/ enhancer binding protein beta
Cdk	Cyclin dependent kinases
CSB	Cockayne syndrome group B
CtBP1-L	C-terminal binding protein 1-L
CtBP1-S	C-terminal binding protein 1-S
DAB	3,3-diaminobenzidine
DAX-1	dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X
DNA	deoxyribonucleic acid
Dpc	days postcoitum
DHEA	dehydroepiandrosterone
DSIF	5,6-dichloro-1-b-D-ribofuranosylbenzimidazole
EF	Elongation factor
ELM-2	Egl-27 and MTA-1 like domain
ER	Estrogen receptor
RNAP II	RNA polymerase II
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GC box	guanine cytosine box
GDP	guanine diphosphate
GTP	guanine triphosphate
HAT	histone acetyltransferase
HCl	hydrochloric acid
HDAC	histone deacetylase
Hmi-er1	human mesoderm induction-early response 1 gene
KLF	kruppel like factors
LCoR	ligand dependent corepressor
LH	lutenizing hormone
LSAB	labeled streptavidin–biotin
LXXLL motif	nuclear receptor box (L= amino acid leucine; X= any amino acid)
M	molar
MAPK	mitogen activated protein kinase
MICoA	MTA1 – interacting coactivator
mi-er1	mesoderm induction-early response 1 gene
mL	milliliter

Mmi-er1	mouse mesoderm induction-early response 1 gene
MPL 1	mouse placental lactogen 1
mRNA	messenger RNA
mta-1	metastasis associated - 1
NAD^+	nicotinamide adenine dinucleotide
N-CoR	Nuclear receptor corepressor
NELF	negative elongation factor
NLS	nuclear localization signal
NaOH	sodium hydroxide
PBS	phosphate buffered saline
PI3 kinase	phosphatidyl-inositol 3 kinase
PLC	phospholipase C
PP II	polyproline II
PPAR	peroxisome proliferators activator receptor
RIP140	receptor interacting protein 140
RNA	ribonucleic acid
RNAPII	RNA polymerase II
RF2a	rice bZIP transcription activator
SANT	SMRT, ADA-2, N-CoR, Transcription factor III
SH3	Src-homology 3
SMRT	silencing mediator of retinoic acid and thyroid hormone
snRNPs	small nuclear ribonucleoproteins
SRC	steroid receptor coactivator
Sp1	promoter specific transcription factor 1
TBP	TATA box binding protein
TFII	transcription factor for RNA polymerase II
TRAP	thyroid hormone receptor-associated protein
VDR	vitamin D receptor
Xmi-er1	*Xenopus* mesoderm induction early response gene 1

1. INTRODUCTION

1.1 General Introduction

1.1.1 Cell proliferation and cancer

A fundamental property of all cells is their ability to grow by synthesizing new proteins, lipids, carbohydrates and nucleic acids. Cell growth must be accompanied by cell division, where one cell gives rise to two new daughter cells. In order for this to occur, the cell must pass through a set of stages, referred to as the cell cycle (Figure 1).

Most cells spend their time in the G1 phase of the cell cycle, where the cell is not dividing (Dictor *et al.*, 1999). Before the cell divides, it must pass through a checkpoint to ensure that the DNA is not damaged and can be replicated properly. After this, the cell enters the S phase, where DNA synthesis occurs and the G2 phase, where the cell continues to grow and produce new proteins. There is another important checkpoint between the G2 and M phases to ensure that the DNA has been replicated properly and that the cell is ready to divide. In the M phase, mitosis and cytokinesis occur, resulting in the division of the cell.

The control of the cell cycle depends on the cyclin dependent kinases (Cdks) and cyclins. Each Cdk protein and its corresponding cyclin protein can form a heterodimer with the Cdk as the catalytic subunit and the cyclin as the regulatory subunit. Thus, by themselves the cyclins have no catalytic activity and the Cdks are inactive without their cyclin partner. The resulting heterodimers can then activate or inactive target proteins to allow entry into the next phase of the cell cycle.

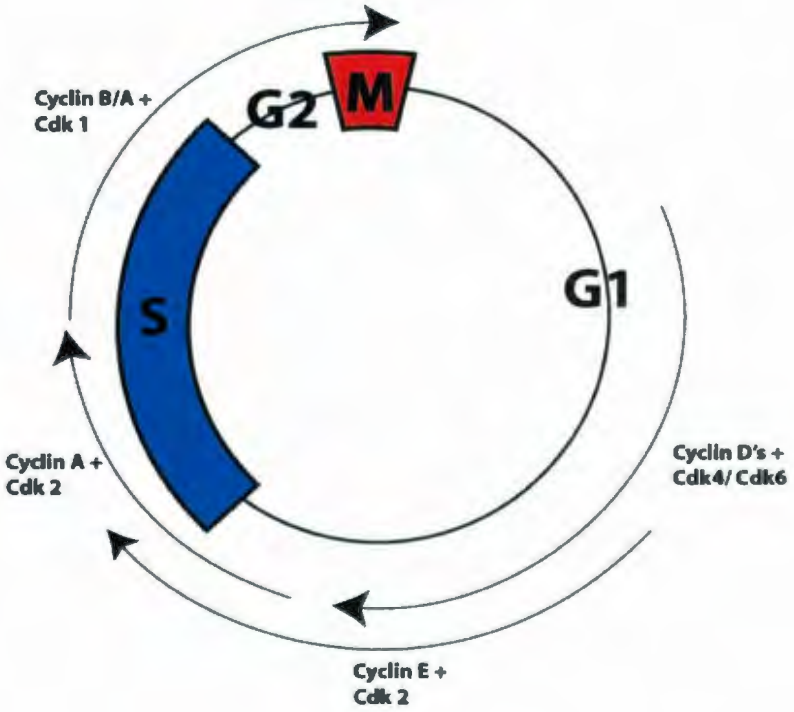


Figure 1: The cell cycle. The cell cycle can be divided into four stages: G1, S, G2 and M. During mid G1 cell cycle activities are dependent on Cdk4 and Cdk6 associations with D-type cyclins. The G1 to S phase transition, where DNA replication is initiated, is driven by the activity of Cyclin E and Cyclin A with the Cdk2 molecule. The G2 to M transition is accomplished by the association of Cyclin B/Cdk1 and Cyclin A /Cdk1 complexes, which may phosphorylate cytoskeletal proteins, histones and nuclear envelope proteins. Finally, the destruction of Cyclin A and B, inactivates Cdk1 and allows the cell to enter back into G1. (Modified from (Lodish, 2000).

The cell cycle machinery may malfunction due to mutations or products of cellular metabolism to result in uncontrolled cell proliferation and the production of a mass of cells, or tumour (Hoeijmakers, 2001). Tumours can be classified as either benign or malignant, depending on their likelihood of spreading. Benign tumours do not invade neighbouring tissues and appear to grow slowly, while malignant (cancerous) tumours can invade or destroy tissues and proliferate rapidly (Cifone, 1982).

Excessive cell proliferation can result from mutations that cause problems in the regulation of the cell cycle. These include two broad types of mutations: oncogenes (gain of function mutations) or tumour suppressor genes (loss of function mutations). An oncogene can arise from the mutation from a normal gene, or proto-oncogene, which are usually components of a growth factor signaling pathway (eg. growth factors, receptors and protein kinases) that promote cell proliferation. The mutations that give rise to oncogenes include point mutations, DNA rearrangements, gene amplifications or chromosome translocations. These mutations cause the gene to be expressed at higher than normal levels, hence the name gain of function mutations. For example the *ras* gene is a proto-oncogene that relays signals from activated receptors on the cell surface by cycling between inactive GDP-bound and active GTP-bound states (Downward, 2003). The activated ras protein activates downstream kinase cascades that ultimately result in cell proliferation. Mutations in this gene result in uncontrolled cell proliferation and tumour formation.

In contrast to oncogenes, tumour suppressor genes suppress cell proliferation by negatively regulating cell cycle progression. Their absence or inactivation can lead to

abnormal cell proliferation and ultimately the growth of a tumour. These genes are the "brakes" that restrain cellular proliferation, whereas oncogenes are the "accelerator". Tumour suppressor genes include the p16 cyclin-kinase inhibitor, which regulates progression through specific stages of the cell cycle.

1.1.2 Transcription

In eukaryotic organisms, protein encoding DNA is transcribed into RNA by the enzyme RNA polymerase II (RNAP II), a 12-subunit polymerase that is able to synthesize RNA and proofread the transcript. The generation of the transcript can be divided into different steps including preinitiation, initiation, promoter clearance, elongation and termination.

The assembly of the preinitiation complex (Figure 2), which consists of the general transcription factors IIA, IID, IIB, IIE, IIF, and IIH; RNA polymerase II; TATA box binding proteins (TBPs) and TATA box associated factors, mark the start of transcription. A mediator, a large protein complex that binds RNAP II and recruits it to the promoter region, is also often required in the preinitiation complex (Blazek *et al.*, 2005).

To initiate transcription, RNAP II and the DNA template need to form an open complex. The open complex is generated by an ATP-dependent process where two general transcription factors (IIE and IIH) melt the double stranded DNA into a single stranded bubble (Goodrich and Tjian, 1994; Holstege *et al.*, 1996; Kim *et al.*, 2000). The start of transcription initiation can be marked by the addition of two initiating nucleoside

triphosphates that are determined by the DNA sequence and the formation of the first phosphodiester bond. The presence of all nucleoside triphosphates and ATP allows RNAP II to clear the promoter and begin elongation (Dvir *et al.*, 1996).

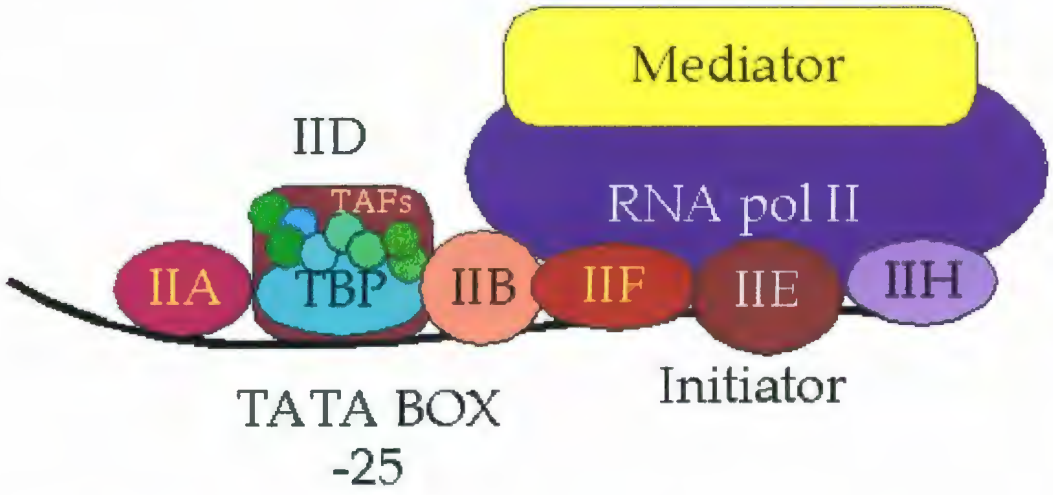


Figure 2: The pre-initiation complex. Transcription factors, IIA, B, D, E, F and H bind to the promoter, along with the TATA box binding protein (TBP), TATA box associated factors. A mediator and RNA polymerase enzyme are also required to initiate transcription. Modified from (Mulligan, 2003)

Before the elongation stage can begin, the promoter must be cleared. During promoter clearance, the promoter initiation complex is partly disassembled. A number of general transcription factors remain at the promoter, such as TFII D, A, H E and mediator, which give structural support for the formation of the next transcription initiation complex (Zawel *et al.*, 1995; Yudkovsky *et al.*, 2000). One of these general transcription factors (TFIIH) is important in facilitating promoter clearance by helping to prevent premature arrest (Goodrich and Tjian, 1994; Dvir *et al.*, 1996; Kumar *et al.*, 1998). Once the promoter is cleared, the elongation process can begin.

Elongation can be defined as the addition of ribonucleosides to a growing mRNA chain (Sims *et al.*, 2004). Elongation is a regulated process that is controlled by various elongation factors (EFs) (any molecule that affects the activities of or is associated with the transcription elongation complex) and cofactors, which are associated with EFs. These factors include TFIIF, elongins (Bradsher *et al.*, 1993a; Bradsher *et al.*, 1993b) and DSIF (5,6-dichloro-1-b-D-ribofuranosylbenzimidazole). They are important in stimulating the rate of transcription by RNAP II. Other elongation factors include NELF (negative elongation factor) and CSB (Cockayne syndrome group B). They play a role in halting RNAP II to allow 5' capping; and modulating the transcription factor TFIIS.

The final step in the transcription cycle is termination. In this step the RNAP II and mRNA strand are released from the DNA. However, before the transcript can be exported from the nucleus for translation, the mRNA needs to be processed (chemical modifications that are necessary to generate a final mRNA product) into a mature mRNA (Proudfoot, 2000). These processes include 5' capping, polyadenylation and splicing.

A. 5' Capping

The process of 5' capping (Figure 3) can be defined as the addition of a guanosine nucleotide that is methylated at position 7 of the purine ring (Shatkin and Manley, 2000). This cap stabilizes the mRNA during translation by binding to ribosomes and translation initiation factors. It is also required to regulate nuclear export and prevent degradation by exonucleases (enzymes that hydrolize nucleic acids).

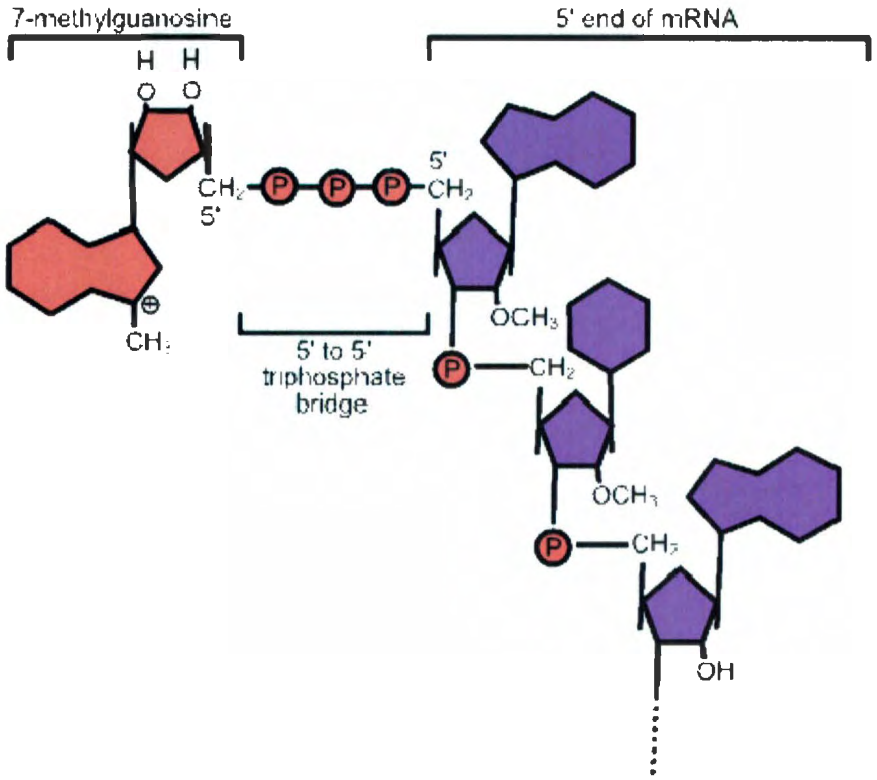


Figure 3: The 5' Cap. The 5' cap is a guanosine nucleotide that is methylated at position 7 of the purine ring. It is added to the 5' end of the mRNA molecule to prevent degradation by exonucleases.

B. Polyadenylation

This process involves the covalent linkage of polyadenylyl moieties (50-250 adenosine nucleotides) to the 3'end of the mRNA molecule by the enzyme poly (A) polymerase (Shatkin and Manley, 2000). The function of the poly (A) tail is to protect the transcript from nuclease digestion and to help export it into the cytoplasm.

C. Splicing

Unlike prokaryotes, the primary mRNA of eukaryotes contains introns (internal parts of the transcript that are not translated) and exons (sequences that appear in the final mRNA and are translated into a protein). To produce a final mRNA product, the introns must be removed and the remaining RNA segments (exons) must be spliced together (Abelson, 1979). This can occur in one of two ways. The main method involves removal of introns by a splicesome, a group of snRNPs (small nuclear ribonucleoproteins) and additional proteins (Chabot and Steitz, 1987). The first step in this method is the formation of a mature splicesome. This occurs when the snRNPs U1 (Zillmann *et al.*, 1987), U2, U4/U6 (Lamond *et al.*, 1988) and U5 bind to the intron. Next, the mRNA is cleaved at the 5' splice site and the newly released 5' end of the intron is covalently joined to an adenine residue at the branch point sequence. This creates a looped structure called a lariat. Finally, the 3' splice site is cleaved and the two ends of the exon are joined together. The excised intron lariat is then targeted for degradation.

In rare cases, there are transcripts that have self-splicing introns (Cech and Bass, 1986). In this instance, the transcript can complete the splicing of introns without the help of any proteins. The intron itself (referred to as a ribozyme) catalyzes this reaction.

1.1.3 Chromatin Structure

Transcription is partially regulated by chromatin structure and the packaging of the DNA molecule (Figure 4). DNA is wound around an octomer of histone molecules (two each of H2A, H2B, H3 and H4) to make up a nucleosome (Becker *et al.*, 2000) . Each histone has a tail of varying length that protrudes from the nucleosome and these tails can be modified (see section B below). The H1 protein then packages the nucleosomes into 30 nm chromatin fibers. The next level of packaging involves the folding of the 30 nm into looped domains, which are approximately 50,000 – 100,000 bp in length. Insoluble networks of non-histone proteins maintain the looped domains. These loops can further be packaged into heterochromatin and finally into chromosomes.

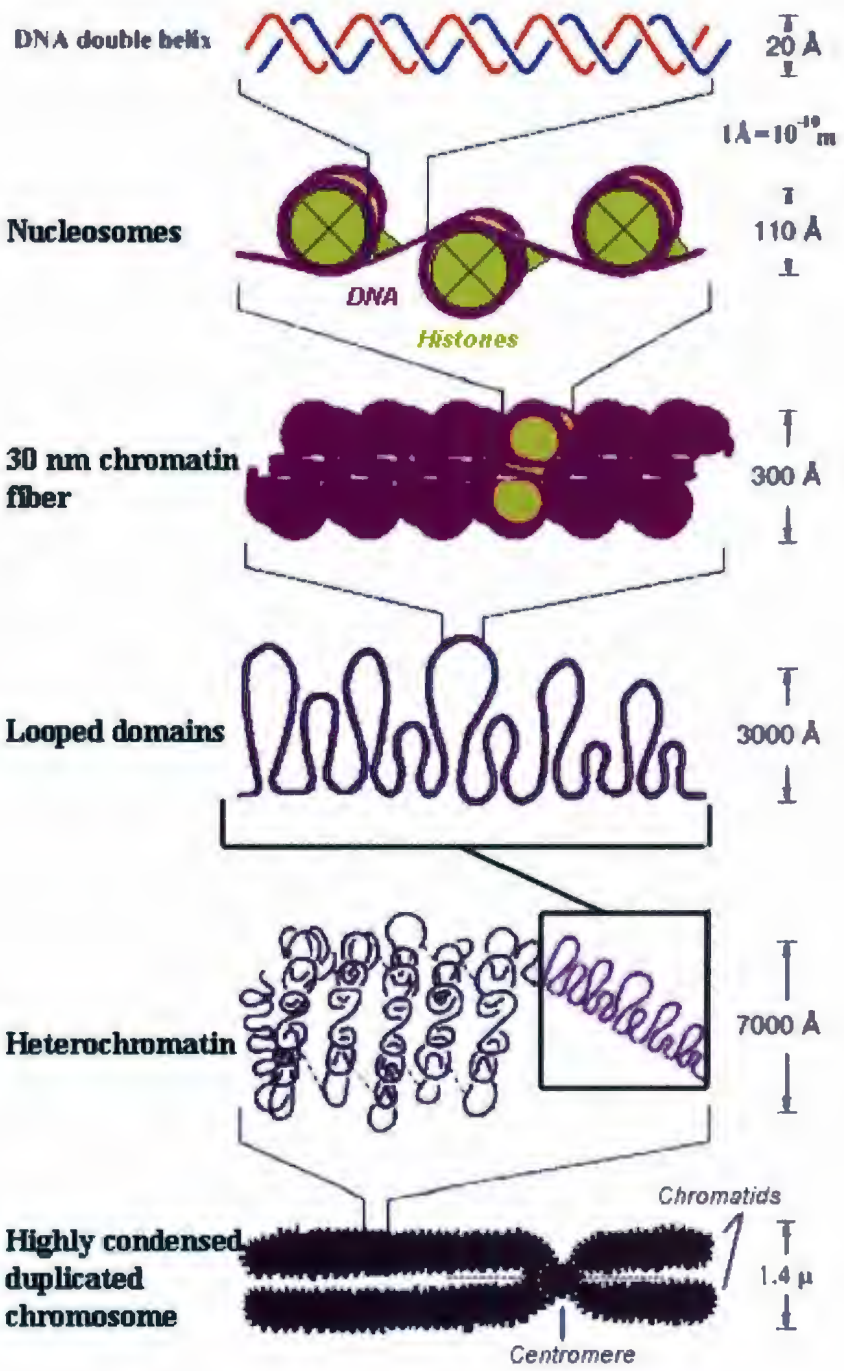


Figure 4: DNA packaging. The DNA molecule is packaged on a number of levels. First, it is packaged into nucleosomes by histone proteins and then into 30 nm fibers. To further condense the DNA, the fibers are packaged into looped domains and then into heterochromatin. Finally, the heterochromatin is packaged into a highly condensed chromosome. (Modified from Becker et al. 2000)

Transcription requires that DNA is accessible to transcription factors and RNA polymerase enzymes, thus, the creation of a stable, tightly-wound, inaccessible chromatin structure can repress transcription, while an unwound, accessible structure can increase the rate of transcription (Narlikar *et al.*, 2002). Two major classes of chromatin-modifying complexes exist: ATP-dependent remodelling complexes and histone acetyl transferase (HAT) or histone deacetylase (HDAC) complexes.

A. ATP-dependent remodelling complexes

These complexes, which consist of an ATPase subunit use ATP hydrolysis to increase the accessibility of nucleosomal DNA. They can move positions, thereby exposing or occluding DNA sequences, and can create conformations where DNA is accessible on the surface of the histone octamer (Narlikar *et al.*, 2002).

B. HAT and HDAC

These complexes covalently modify nucleosome structure by acetylating or deacetylating lysine residues in the N-terminal region of the histone proteins. The enzyme HAT acetylates conserved lysine residues on the amino terminal tails of core histones (Davie, 1998), thereby opening the chromatin structure and increasing the rate of transcription. They include two broad classes: nuclear (A-type) and cytoplasmic (B-type) HATs, with further subdivisions within each class. HDACs have the opposite effect; they deacetylate lysine residues at the N-terminus of histones, condensing the chromatin, making it inaccessible to transcription factors, and ultimately repressing transcription.

Phylogenic analyses have subdivided HDACs into four families:class I, II, III (sirtuins) and IV. The catalytic domain of classess I, II and IV share sequence and structural homology and all require zinc for their catalytic mechanism. In contrast, the sirtuins (class III) do not share sequence or structural homology with the other HDAC classes and use a distinct catalytic mechanism that involves the cofactor nicotinamide adenine dinucleotide (NAD+) (Frye, 2000).

1.2 Mesoderm induction - early response gene 1

1.2.1. Fibroblast growth factors and mesoderm induction

Fibroblast growth factors (FGFs) are signaling proteins that are important in cellular responses, including mitogenesis, differentiation, angiogenesis and transformation (Baird and Klagsbrun, 1991). There are at least 19 different members present in the FGF family, which are related by amino acid sequence and their ability to bind heparin (Miyamoto *et al.*, 1993). The FGFs bind to FGF receptors (FGFR), a subfamily of cell surface receptor tyrosine kinases, present on the cell membrane and this action induces responses in the cell. High affinity FGFRs (Figure 5) are transmembrane proteins that have an extracellular binding domain consisting of two to three immunoglobulin domains. They have an intracellular domain that consists of a juxtamembrane region, a tyrosine kinase domain and a carboxyl terminal tail (Jaye *et al.*, 1992). When FGFs bind to the extracellular binding domain of the receptor, the receptor becomes dimerized to trigger tyrosine kinase activation (McKeehan *et al.*, 1998), which leads to autophosphorylation of the intracellular domain. The autophosphorylation of

tyrosine acts as a mechanism for the construction and recruitment of signalling complexes (Schlessinger, 2000). Signaling proteins possessing protein-protein binding motifs such as Src homology 2 domains can then bind to the phosphorylated tyrosines, which in turn leads to the phosphorylation and activation of these signalling proteins (Pawson *et al.*, 1993; Forman-Kay and Pawson, 1999). The signal transduction cascade can then occur through either of three pathways: the Ras/MAPK pathway, the PLC gamma/Ca^{2+} and the PI3 kinase/Akt pathway.

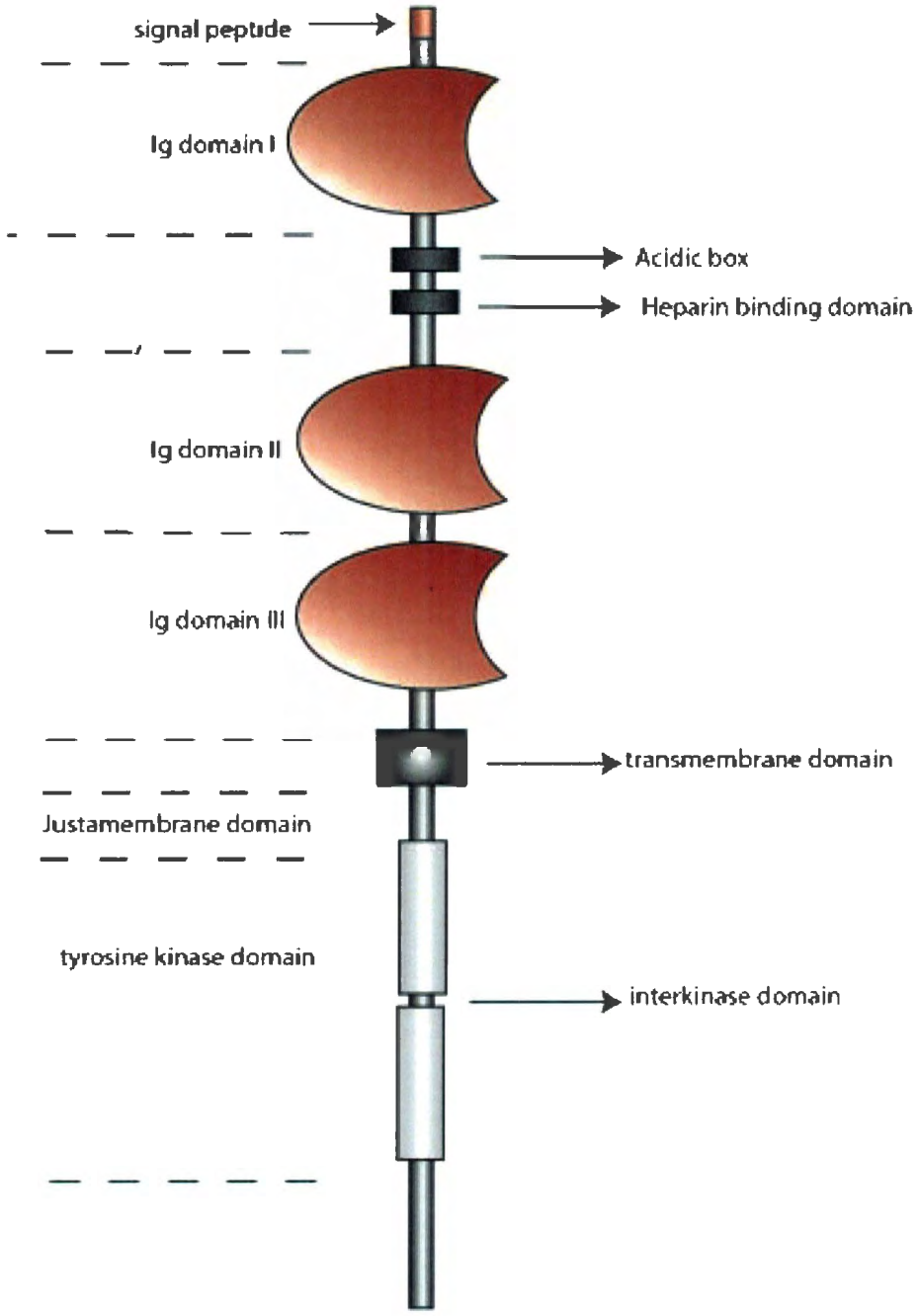


Figure 5: The fibroblast growth factor receptor. The FGFR consists of a signal peptide, three immunoglobulin domains (which are separated by an acidic box), a transmembrane and juxtamembrane region, and a tyrosine kinase domain (which is interrupted by an interkinase domain). These domains have different functions that allow signaling through this receptor.

The differentiation of the mesoderm in *Xenopus laevis* embryo explants is induced by FGFs (Slack *et al.*, 1987). The mesoderm arises from cells located in the equatorial region of the blastula stage embryo and these cells are induced to form mesoderm when a signal is released from neighbouring vegetal cells (Nieuwkoop, 1985) (Figure 6). During mesoderm induction, FGF binds to receptors in the cell membrane, which become phosphorylated on tyrosine. This then binds to intracellular substrates to form a signaling complex (Ryan and Gillespie, 1994). This results in the activation of a number of signal transduction pathways. For example, protein kinase C (Gillespie *et al.*, 1992) and a mitogen-activated protein kinase become activated during mesoderm differentiation (Hartley *et al.*, 1994). The ultimate targets of the signal transduction pathways are the immediate early genes. These cellular genes are expressed immediately after resting cells are stimulated by extracellular signals such as growth factors and neurotransmitters.

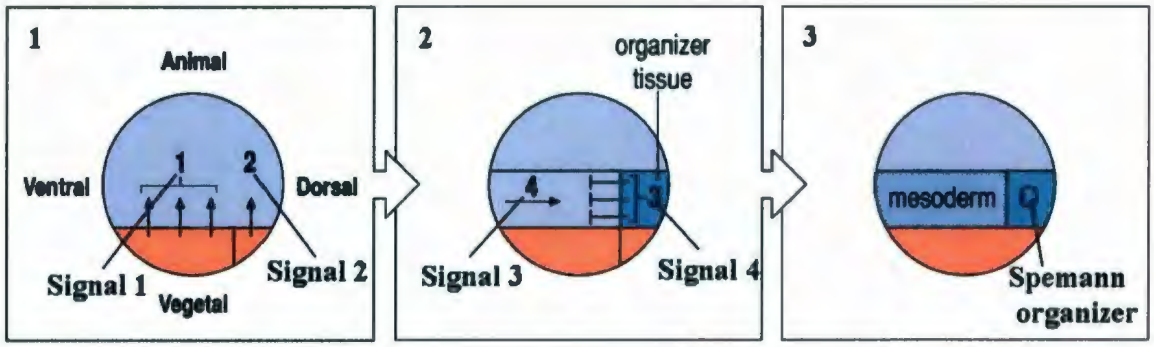


Figure 6: Mesoderm induction in *Xenopus* embryos. The first two signals for mesoderm induction are released from the vegetal pole – one from the ventral side and one from the dorsal side. The ventral mesoderm is specified by signal 1, while the Spemann organizer and the dorsal mesoderm are specified by signal 2. Signal 3 dorsalizes the adjacent mesoderm by inhibiting the ventralizing action of signal 4. Modified from (Wolpert, 2002)

1.2.2. *Xenopus mi-er1*

Many studies using *Xenopus laevis* as a model system have been important in elucidating molecular mechanisms of gene regulation and for studying the earliest stages of vertebrate embryonic development due to its external development and the large number of embryos that it produces. The *mi-er1* gene was first identified in *Xenopus laevis* embryo explants that were being induced to differentiate into mesoderm (Paterno *et al.*, 1997). *Xmi-er1* contains an open reading frame of 1497 base pairs that is predicted to encode a protein of 493 amino acids. The predicted protein contains one functional nuclear localization signal (NLS), targeting it to the nucleus (Post *et al.*, 2001) as well as stretches of acidic amino acids in its N-terminus, that have been shown to function as a transcriptional activator (Paterno *et al.*, 1997). *Xmi-er1* is similar in three regions to the rat metastasis-associated gene, *mta-1*, a gene whose expression is indicative of a metastatic phenotype. *Mta-1* and *mi-er1* are not homologs, but their gene products are possibly related families of proteins or proteins that contain similar domains.

The gene *mi-er1* is to be considered an immediate early gene (a gene that is activated transiently and rapidly by cellular stimuli at the transcription level in the first round of response to stimuli, before new proteins are synthesized) for many different reasons. First, fibroblast growth factor-induced increase in *mi-er1* is not dependent on *de novo* protein synthesis, since the protein inhibitor, cycloheximide does not prevent FGF induced increases in MI-ER1 levels (Paterno *et al.*, 1997). Furthermore, *Xmi-er1* transcripts are predominant during initial cleavage and blastula stages in *Xenopus*

development, and they peak during late blastulation. This evidence, taken together, shows that *mi-er1* is an immediate early gene.

1.2.3 Human *mi-er1*

Human *mi-er1* is a single copy gene, located on chromosome 1, spanning 63 kilobases and containing 17 exons (Paterno *et al.*, 2002). Exon 3A is a "skipped exon", that is included in the transcript only at certain times. The last intron functions as a facultative intron, an intron that is also only present in the mature transcript at certain times. Three distinct 5' ends have been identified for the transcripts of this gene, which either include exon 1A or 1B while the skipped exon 3A is alternatively used. The other exons (exons 4 to 15) are constitutive exons that are always included in the transcript. There are also four distinct 3' ends that have been identified, which have been called *a, bi, bii,* and *biii*. These depend on the alternate inclusion of the facultative intron (intron 15) and the alternate use of three polyadenylation signals (Figure 7).

The different transcripts code for proteins with different N and C termini. The three different 5' ends encode for 3 distinct N terminal domains (Paterno et al. 2002). The first N terminal domain (N1) contains exon 1A, exon 2A and exon 3A. Another N-terminal domain (N2) consists of exon 1A and exon 2A only. Finally, another N terminal domain (N3) has exon 1B instead of exon 1A and does not contain exon 3A. The four alternate 3' ends encode two distinct C-terminal domains: alpha and beta. The alpha C terminus results from the removal of intron 15 and encodes a 23 amino acid domain. The beta C-terminus represents a sequence encoded by the *bi, bii* and *biii* ends and includes

intron 15. The three distinct N-termini in combination with the two C-termini are predicted to encode six distinct human MI-ER1 protein isoforms. These are multiple forms of the same protein that differ in their amino acid sequence and are produced by alternative splicing of mRNA. Thus, the six different isoforms of *mi-er1* are N1 alpha, N1 beta, N2 alpha, N2 beta, N3 alpha and N3 beta (Figure 7). These are 457, 536, 432, 511, 433 and 512 amino acids respectively. Although, six different protein isoforms have been found in the human, only the N3 beta isoform has been found in *Xenopus to* date.

The protein hMI-ER1 displays 91% similarity to the xMI-ER1 protein with stretches of 100% identity. These include one NLS and a proline rich region corresponding to the consensus for binding Src-homology 3 (SH3) domains. A SANT domain, which is characteristic of proteins involved in transcriptional regulation, is also 100% conserved between the human and *Xenopus* MI-ER1. This high degree of identity between human and *Xenopus* MI-ER1 indicates that it is conserved between vertebrate species.

Human *mi-er1* is expressed at very low levels in normal human tissues, but *mi-er1* mRNA expression is upregulated in breast carcinoma cell lines and in breast tumours (Paterno *et al.*, 1998). This indicates that the expression of *mi-er1* is associated with the neoplastic state (abnormal and uncoordinated tissue growth) in human breast carcinomas. Therefore, *mi-er1* may play a role in the pathology of human cancer.

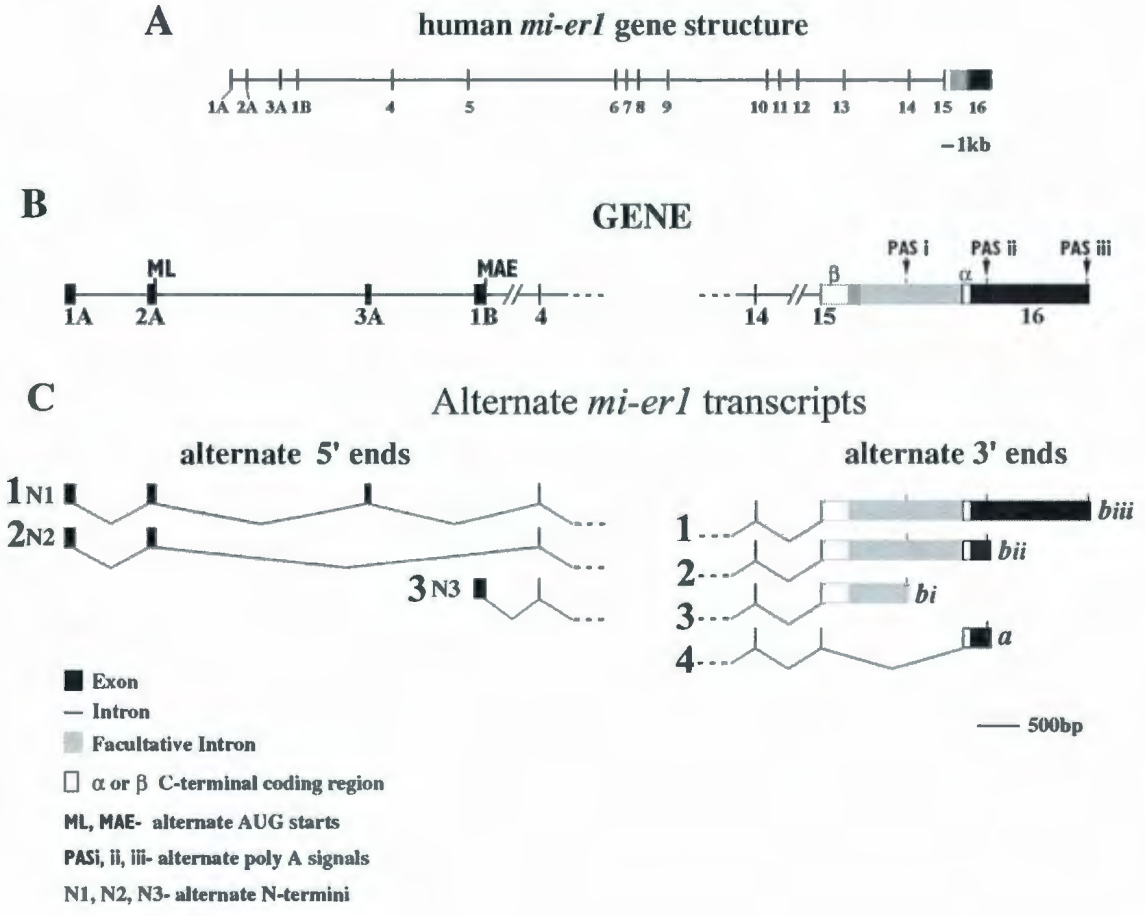


Figure 7: Structure of human *mi-er1* gene and its transcripts (adapted from Paterno *et al.* 2002)

The structure of the mi-er1 gene (A) and a magnified version (B) shows the arrangement of the exons, introns, C-terminal coding regions, and alternate AUG start sites. The alternative transcripts (C) have 3 alternative 5'ends and 4 alternative 3' ends that arise as a result of alternative splicing, alternative start sites and the use to alternative poly A signals.

1.2.4 Mouse *mi-er1*

Recently, the mouse ortholog of *mi-er1* was cloned and characterized as a single copy gene located on chromosome four (Thorne *et al.*, 2005). Mouse *mi-er1* has the same intron-exon structure as the human gene with the exception of exon 3A, in which an alternate upstream 3' splice acceptor is utilized. Like the human version, multiple transcripts are produced in the mouse, including orthologs of human N1 beta, N2 beta, and N3 beta. A novel mouse isoform has been isolated containing sequence from an additional exon located between exon four and five that produces a fourth alternate N-terminus. The MI-ER1 alpha transcripts have been detected in the testis at the RNA level by a Northern blot (Grant, 2004). N1 beta and N3 beta are ubiquitously expressed in the mouse, while N4 beta is only expressed in the testis. N2 beta is expressed in most tissues, but is not found in heart, brain, eye, or skeletal muscle (Thorne *et al.*, 2005).

The mouse *mi-er1* gene is currently being studied since the mouse (*Mus musculus*) has the potential to make major contributions to the study of *mi-er1*. The study of the structure and expression of mouse *mi-er1* is necessary for any future construction of transgenic mice, whose genetic constitution has been experimentally altered by the addition or substitution of genes (Randall D., 2002). This type of study could be extremely useful to the study of *mi-er1* and its function in human cancers as the human and mouse genes are more than 98 percent identical. Thus, by determining the function of *mi-er1* in the mouse, important information can be learned about *mi-er1* in humans.

1.2.5 Domains of MI-ER1

A. SANT domain

The MIER-1 protein contains a SANT domain. This domain has been implicated in DNA binding and in protein-protein interactions (Aasland *et al.*, 1996), which include interactions with complexes containing histone deacetylase (Guenther *et al.*, 2001; You *et al.*, 2001). This domain has previously been found in other transcription regulatory molecules such as SMRT (Ordentlich *et al.*, 1999), N-CoR (Aasland *et al.*, 1996) and in transcription/chromatin regulatory complexes such as MTA-1 (Toh *et al.*, 2000). Proteins that are involved in cell differentiation and development may contain the SANT domain as well. For example, the protein Eg1-27 found in *C. elegans* (Solari *et al.*, 1999), and the protein CoRest (You *et al.*, 2001) both contain the SANT domain.

The SANT domain of hMI-ER1 has recently been shown to bind and inhibit the Sp1 protein, a transcription factor with three zinc-fingers that binds GC-boxes and assists the further binding of the multiprotein complex TFIID, which promotes the initiation of gene transcription (Dynan and Tjian, 1983; Kolell and Crawford, 2002; Yoo *et al.*, 2002; Yu *et al.*, 2003). HMI-ER1 physically associates with the Sp1 protein, through its SANT domain, thereby preventing Sp1 from binding to the promoter and activating gene transcription (Ding *et al.*, 2004). Thus, by binding to the Sp1 protein through the SANT domain, hMI-ER1 can repress transcription of target genes.

B. ELM-2 domain

MI-ER1 contains the ELM-2 domain, which is N-terminal to its SANT domain. It has been found that MI-ER1 recruits histone deacetylase (HDAC) to its ELM-2 domain (Ding *et al.*, 2003). HDAC is an enzyme that removes an acetyl group from N-acetyl lysine amino acids on a histone protein. Deacetylation restores a positive charge, increasing histones affinity for DNA and consequently down regulating transcription. Thus, by recruiting HDAC to its ELM-2 domain, hMI-ER1 acts a transcriptional repressor.

C. NLS

The MI-ER1 protein sequence also contains one functional NLS (Post *et al.*, 2001), short stretches of amino acids that mediate the transport of proteins into the nucleus (Tinland *et al.*, 1992). The only functional NLS in the MI-ER1 protein is located in the beta C-terminal domain. Thus, only the beta isoform can be transported to the nucleus by its NLS. Although alpha does not contain a NLS, binding to other proteins that contain these sequences can transport it to the nucleus.

D. Acidic Activation Domain

The MIER-1 protein contains four acidic activation domains that have been previously characterized for the xMI-ER1 protein (Paterno et al. 1997). Acidic activation domains are found in many transcription factors, such as the protein RF2a (bZIP transcription factor, which regulates expression from the promoter of rice tungro

bacilliform bandavirus) (Dai *et al.*, 2003). These domains function to activate transcription of other genes.

E. Proline-rich region

The MIER-1 sequence also contains a proline rich region near the C-terminus. Proline rich regions are common binding motifs because they contain PP II helices, which are extended structures with three residues per turn (Kay *et al.*, 2000). The proline residues in the PP II helix form a continuous hydrophobic region around the surface of the helix. The backbone carbonyl groups are ideal hydrogen bonding sites, since they are conformationally restricted, making them poorly hydrated and electron-rich. Therefore, PP II helices present an easily available hydrophobic surface, as well as a good hydrogen-bonding site.

The MI-ER1 protein contains a proline rich motif in its common region, C-terminal to the SANT domain. It has been shown that the proline 365, within the proline rich region, regulates the activity of XMI-ER1 and inhibits mesoderm induction in *Xenopus* embryonic development (Teplitsky *et al.*, 2003).

F. LXXLL motif

The MI-ER1 alpha protein contains an LXXLL motif, where L represents the amino acid leucine and X represents any other amino acid. This motif (also referred to as the NR-box) is commonly found in nuclear receptor coactivators (SRC-1, CBP, MICoA).

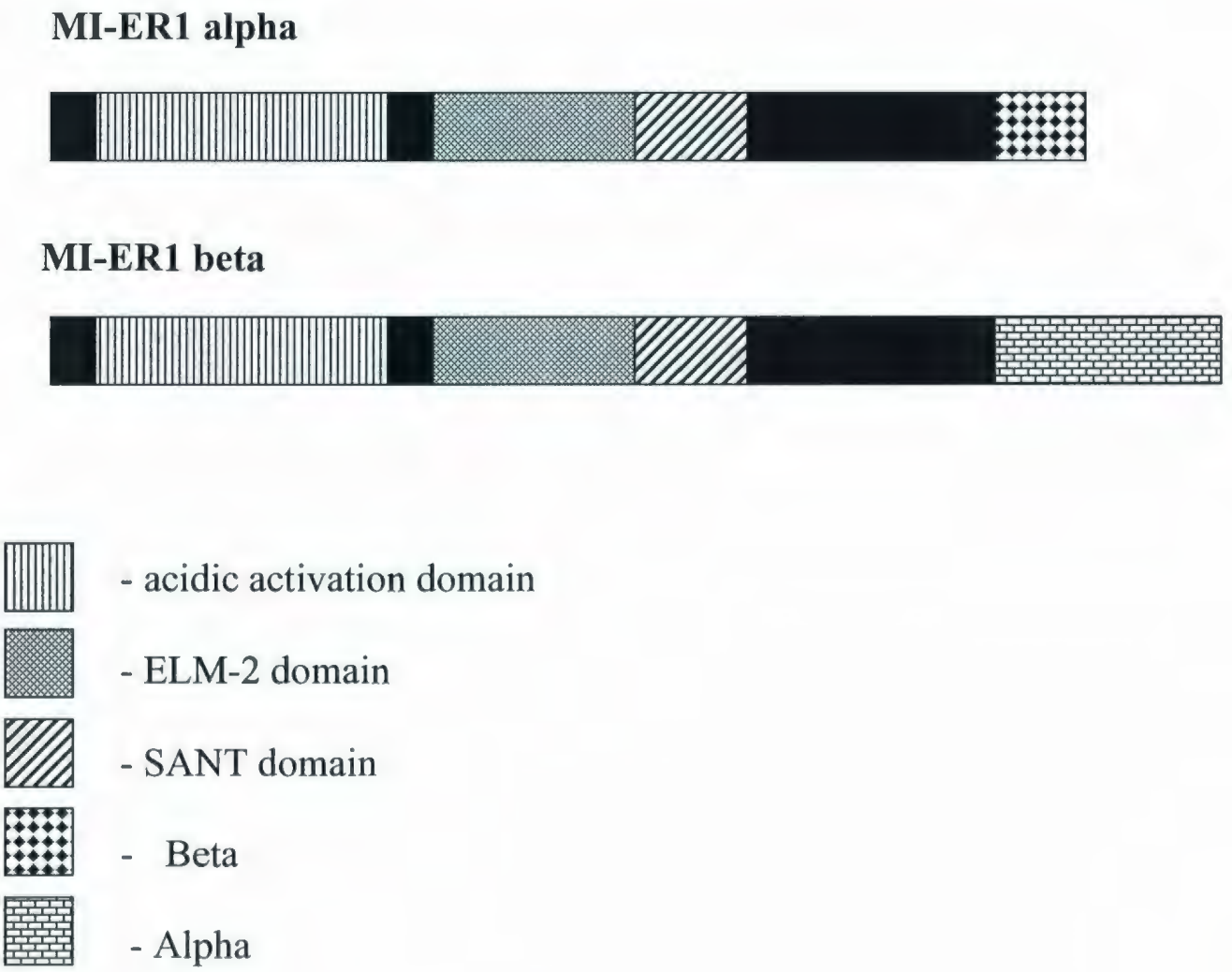


Figure 8: Domains of the MI-ER1 protein. MI-ER1 contains many different domains. Alternate splicing gives rise to two isoforms that differ in the C-terminal (alpha and beta). Black boxes indicate areas where there is no known domain.

1.2.6 Subcellular localization of MI-ER1

In this study, the subcellular localization of the MI-ER1 protein in mouse tissues was examined. It is important therefore to note which isoform contains the NLS and where the isoforms are located in *Xenopus* and human.

The MI-ER1 beta isoform contains the only functional NLS in its C-terminal domain, which targets it to the nucleus. However, in *Xenopus*, MI-ER1 beta is retained in the cytoplasm during cleavage stages by binding to a cytoplasmic anchor (Post *et al.*, 2005) and only begins to appear in the nucleus at mid-blastula stage (Luchman *et al.*, 1999). Transfection assays have revealed that hMI-ER1 alpha, which lacks the NLS, remains in the cytoplasm (Paterno *et al.*, 2002). These studies show that MI-ER1 beta is found primarily in the nucleus, while MI-ER1 alpha is found in the cytoplasm.

1.3 Mouse embryo staging

In this study, the expression of MI-ER1 was examined in 8, 12 and 16-day mouse embryos, thus it is important to understand the development of the embryo at these stages.

The 8-day embryo is the first stage where MI-ER1 expression was examined in this study. At this point in development, (Figure 9) the brain plate of the central nervous system has developed rapidly in the mouse and determines the form of the embryo (Theiler, 1972). Furthermore, at this stage the first seven somites appear, with the neural folds closing at the level of the fourth and fifth somite. At this age, the circulatory system develops by the formation of blood islets (an aggregation of mesodermal cells on

the embryonic yolk sac that can potentially form the vascular endothelium and primitive blood cells) and the endocardial tube. Within the circulatory system, the heart rudiment and pericardial cavity develop and the dorsal aorta starts to form. At 8-days of age, the foregut pouch appears and the gut epithelium becomes columnar and cuboidal in certain areas.

By the time the embryo is 12 days old, it has taken on a much different shape (Figure 9) and is seven to nine millimeters long (Theiler, 1972). The central nervous system becomes more developed at this stage, with the appearance of the pineal gland, thickening of the lens vesicle and development of the semicircular canals. The heart also develops further: the atrium becomes septated and the truncus arterious (located between the pulmonary artery and aorta) begins to part. At this stage the lungs are buds, which have developed both secondary and tertiary bronchi. Finally, the epithelium of the digestive tract also becomes specialized in the different regions and there is blood formed in the liver.

The embryo develops greatly by the time it becomes16 days old (Figure 9). It has grown to 14-17 mm, the fingers and toes have formed, the eyes develop, the auditory meatus is covered by pinna and the abdominal cavity has enlarged (Theiler, 1972). Within the central nervous system, the primary cerebral cortex enlarges, and in the diencephalon the hypophysis begins to differentiate from the pineal gland. The heart and vessels of the circulatory system also have their final prenatal configuration. Furthermore, the intestine forms crypts and the blood cell production in the liver increases. Finally, the lung tissue becomes compact and more vascularized.

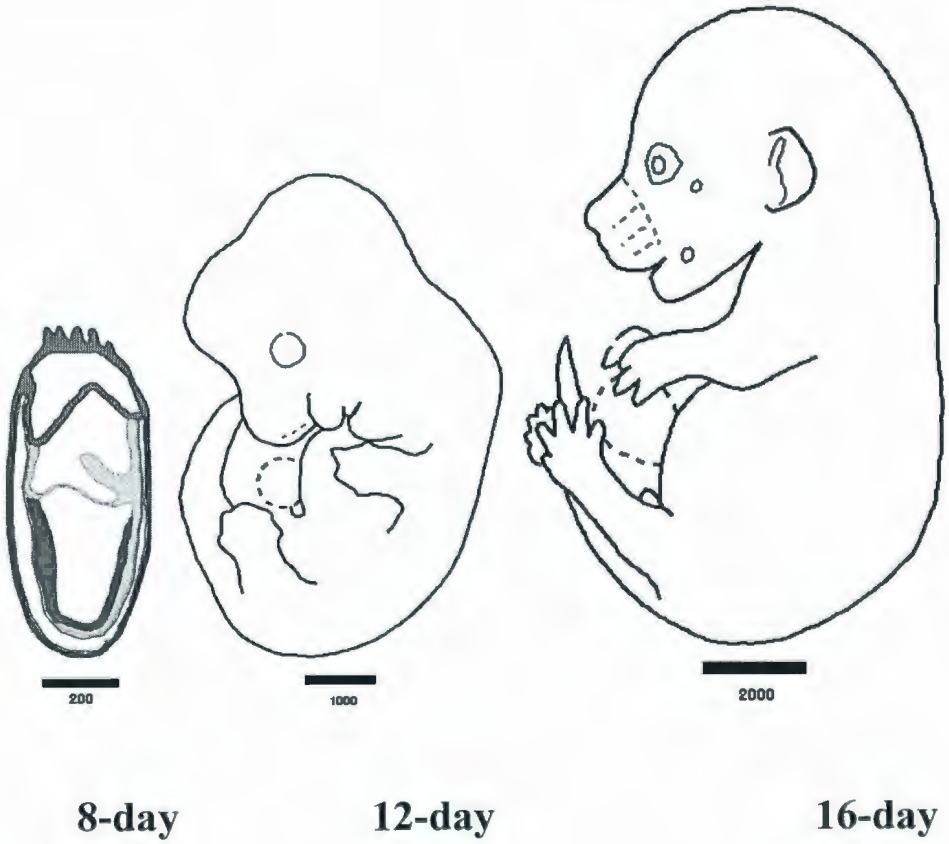


Figure 9: General Overview of Mouse Embryo Development from 8 to 16 days. From left to right: 8-day old embryo, 12-day old embryo, and 16-day old embryo (modified from Downs and Davies, 1993).

1.4 Objective of study

The purpose of this study was to determine the expression of the MI-ER1 protein in various embryonic stages and in different adult tissues of the mouse by immunohistochemistry and to determine the subcellular localization of the MI-ER1 alpha and beta proteins. Expression patterns are important to the study of proteins as they can provide an indicator of where the protein may function and when it is functioning in a particular tissue. Thus, this expression study will give us important information regarding MI-ER1 in different mouse tissues.

The *mi-er1* gene has been cloned and characterized in *Xenopus*, human, mouse and functional studies of the MI-ER1 protein have been completed *in vitro* and *in vivo* in various cell lines. However, to date there has been no work done with *in vivo* mouse models. Thus, a major goal in the MI-ER1 study is to construct transgenic mice. Before such a major project is started, however, preliminary work must be completed to investigate where and when the MI-ER1 protein is expressed in the mouse embryo and adult. This study will also aid in deciding what type of transgenic mouse would be most useful to construct in the future.

2. MATERIALS AND METHODS

2.1 Mouse embryo and adult tissue sections

Mouse adult brain, adrenal gland, heart, kidney, liver, lung, ovary, pancreas, leg muscle, intestine, spleen, testis, and all the embryo sections were obtained from Novagen, EMD Biosciences (Mississaga, Ontario, Canada). The tissue or embryos were dissected from the mouse and immersed immediately in PBS containing four percent paraformaldehyde. After fixation for twenty-four hours at four degrees Celsius, the tissues were washed in PBS, dehydrated through a graded series of ethanol washes, cleared in xylene and embedded in paraffin. Sections were then cut at a thickness of seven microns and mounted on slides that had been previously treated for adherence. The slides were shipped at ambient temperature and stored at four degrees Celsius before use.

Mouse adult thyroid sections were obtained from Zyagen Laboratories (San Diego, California, USA). The organs were freshly harvested from Swiss Webster mice and cut into small pieces, fixed for sixteen to twenty-four hours in ten percent neutral buffered formalin and embedded in paraffin. The paraffin blocks were sectioned at a thickness of five to seven microns and mounted on slides treated for adherence. The sections were shipped at room temperature in tightly closed slide boxes. (Note: Either Novagen EMD Biosciences or Zyagen completed the tissue fixation, preparation, embedding and sectioning).

2.2 Immunohistochemistry using anti pan MI-ER1

Immunohistochemistry was performed using the Vectastain Elite ABC kit from Vector Laboratories (Burlington, Ontario, Canada). First, the sections were deparaffinized in three changes of xylene for five minutes and hydrated through a graded alcohol series (two changes of 100 percent alcohol for five minutes each, one change of ninety-five percent alcohol for one minute and one change of seventy percent alcohol for one minute). The sections were then rinsed with water to remove any alcohol and to completely hydrate the tissues. To quench any endogenous peroxidase activity, the sections were incubated in zero point three percent hydrogen peroxide for thirty minutes. After this, the sections were rinsed in distilled water to remove excess hydrogen peroxide.

When tissues are fixed and processed, the antigens often become masked and need to be retrieved. To retrieve the antigen, the sections were incubated in a ten millimolar sodium citrate buffer. The buffer was first heated in the microwave to ninety-five degrees Celsius in a plastic coplin jar, the slides were then placed in the buffer and incubated in a ninety-five degrees Celsius water bath for ten minutes. After removal from the water bath, the sections were cooled to room temperature while in the buffer and then rinsed in PBS for five minutes.

To block the sections from non-specific labeling, the sections were incubated in one percent goat serum (Vector Laboratories) in PBS for twenty minutes and the slides were blotted around the section with gauze after the incubation time. After this, the antibody was appropriately diluted in one percent BSA in PBS and one milliliter of the

diluted pan MI-ER1 antibody (Paterno *et al.*, 2002) (1:800 dilution) was applied to each slide. The slides were then placed in a covered chamber to prevent evaporation and placed at four degrees Celsius overnight

The following day, the slides were washed with PBS for five minutes to remove excess antibody. Next, the sections were incubated in diluted biotinylated goat anti-rabbit secondary antibody for thirty minutes and the slides were rinsed in PBS. Vectastain Elite ABC reagent (Vector Laboratories) was then added to the slides and they were incubated for another thirty minutes. After the incubation, the slides were rinsed in PBS for five minutes and then the slides were incubated with DAB from Dako (Mississago, Ontario, Canada) until the desired staining intensity developed (between 45 seconds and two minutes). The slides were then rinsed in distilled water.

To counterstain the sections, the slides were incubated in hematoxylin for three minutes, rinsed, and differentiated in one percent acid alcohol (dipped in solution for approximately three seconds). After rinsing again, the sections were blued with Scott's tap water for one minute and rinsed in distilled water. To dehydrate the sections, the slides were incubated in seventy percent alcohol for one minute, ninety-five percent alcohol for one minute and two changes of one hundred percent alcohol for three minutes each. The sections were then cleared in two changes of xylene for five minutes each and mounted in Permount media. The sections were viewed under a compound light microscope (Olympus BH-2) and the pictures were taken with a digital camera.

To analyze the sections, the intensity of staining and the percent of nuclei stained were examined. The intensity of staining was graded semi-quantitatively by three

separate people (myself, Dr. Laura Gillespie and Ph.D. student Patricia McCarthy) according to a scale of: + = weak staining, ++ = moderate staining, and +++ = strong staining. An estimated value for percent of nuclei stained in each tissue was also examined qualitatively by viewing the tissue under low power (forty times magnification) on the light microscope.

2.3 Immunohistochemistry using anti-MIER1 alpha

Immunohistochemistry was performed using the Universal LSAB kit (Dako). The sections were cleared, hydrated, and quenched of peroxidase activity as described for the anti-pan MI-ER1. The antigen retrieval method was also performed the same as described for anti-pan MI-ER1.

To block the sections from non-specific labeling, the sections were incubated in universal blocker (Dako) for twenty minutes and the slides were blotted around the section with gauze after the incubation time. Following this, the MI-ER1 alpha antibody (Paterno *et al.*, 2002) was appropriately diluted in one percent BSA in PBS and one milliliter of the diluted antibody was applied to each slide. The slides were then incubated overnight at four degrees Celsius. The method of incubation was performed as described for the previous antibody, with the exception of the antibody dilution which was 1:1200.

The following day, the slides were washed with PBS for five minutes to remove excess antibody. Next, the sections were incubated in biotinylated anti-rabbit secondary

antibody in PBS (Dako) containing stabilizing protein and 0.015 molar sodium azide for thirty minutes and the slides were rinsed in PBS. Strepavidin conjugated to horseradish peroxidase in PBS (Dako) containing stabilizing proteins and an antimicrobial agent was then added to the slides and they were incubated for another thirty minutes. After the incubation, the slides were rinsed in PBS for five minutes and then the slides were incubated with DAB (Dako) until the desired staining intensity developed (between forty five seconds and two minutes). The slides were then rinsed in distilled water.

Counterstaining, dehydration, clearing, mounting, photography and analysis were performed as described for the previous antibody.

2.4 Immunohistochemistry using anti- MI-ER1 beta

Unfortunately, anti-MI-ER1 beta was not suitable for immunohistochemistry of these prepared sections. Although the antibody is suitable for Western blotting and immunoprecipitations, it gave an unsuitable amount of background staining when the preimmune serum was used. Therefore, we can only deduce the expression of the beta isoform until an appropriate antibody becomes available. Since mMI-ER1 has two distinct C-termini, it is possible to deduce expression of the beta isoform by analyzing the staining of the mouse tissues with the pan-antibody (recognizes both isoforms) and comparing it to the staining with the alpha antibody. The only time where we can definitely conclude that MI-ER1 beta is expressed is when there is staining with the pan, but not the alpha antibody. When there is staining with both the pan and the alpha

antibody, we can conclude that MI-ER1 alpha is expressed, but we cannot determine whether MI-ER1 beta is expressed.

3. RESULTS

3.1 MI-ER1 expression in mouse embryonic and corresponding adult tissues

The 8, 12, and 16-day embryonic stages cover a major part of mouse development given that the average gestation time is 20 days. Therefore, 8, 12 and 16-day mouse embryos, as well as adult heart, lung, liver, intestine, brain and skeletal muscle were stained with both pan anti-MI-ER1 (1:800 dilution) and anti MI-ER1 alpha (1:1200 dilution). Also, the expression of the beta isoform can, in some instances, be deduced from the expression pattern found using the alpha and the pan antibody. The expression of MI-ER1 beta can only be deduced when expression is detected with the pan antibody, but not the alpha antibody. For example, if the pan antibody detects expression in the nucleus and the cytoplasm and the alpha antibody detects expression in the cytoplasm, then we can definitely conclude that beta is expressed in the nucleus. It may also be expressed in the cytoplasm with alpha, but we would need a beta specific antibody to definitely conclude this.

The immunohistochemical analysis revealed ubiquitous nuclear and cytoplasmic expression in cells of the 8, 12, and 16-day embryo when stained with the pan antibody. There was no expression detected with the alpha antibody, thus, we can conclude that only MI-ER1 beta is expressed in the 8, 12 and 16-day mouse embryonic cells.

3.1.2 Brain

There was intense expression of MI-ER1, both in the cell nuclei (75% of nuclei) and in the cell cytoplasm in embryonic brain cells shown by using the pan anti-MIER1 antibody (Figure 10 C and E). Expression was not detected with anti-MI-ER1 alpha (Figure 10 G and I), indicating that the beta isoform is only expressed. Therefore, MI-ER1 beta is expressed in the cell nuclei and cytoplasm of the embryonic brain.

In the adult brain, a complex pattern of staining was observed and the level of staining was significantly decreased compared to the embryo. Pan anti-MI-ER1 detected a moderate level of cytoplasmic expression in cells of the cortex region of the brain, particularly in the pyramidal cells (Figure 11C and D). There were certain areas of the cortex where the pyramidal cells were moderately stained (75%), while there were other areas where little staining was detected. Weak cytoplasmic expression of MI-ER1 alpha was detected with the anti-MI-ER1 alpha antibody in the pyramidal cells (Figure 12C and D), indicating that MI-ER1 alpha is expressed here. Thus, MI-ER1 alpha and possibly beta are expressed in the cytoplasm of the pyramidal cells. In addition, there was moderate level intensity staining of the neuronal processes in the cortex with the alpha specific antibody.

Expression was detected in the hippocampus of the brain (Figures 11 G/H and12 G/H). Exclusive and moderate cytoplasmic expression was found in the CA3 cells (Figure 11 H and 13 H) with both the pan and the alpha antibody, indicating that the alpha isoform (and possibly beta) are expressed here.

In the thalamus (Figures 13 C/D and 14 C/D), there were varying levels of cytoplasmic staining in cells with the pan and alpha antibody. In some areas there was intense cytoplasmic staining of the pyramidal cells (60%), while there were other areas where no staining was detected. Given the cytoplasmic staining with both the pan and alpha antibody, MI-ER1 alpha and possibly beta are expressed in this region of the brain.

The alpha antibody detected an intense level of MI-ER1 alpha expression in the cytoplasm of the neurons in the hypothalamus; the pan antibody detected only weak expression (Figures 13 G/H and 14 G/H). This shows that the alpha isoform (possibly beta) are present in the cell cytoplasm. Furthermore, there was also a moderate level of anti-MI-ER1 alpha staining in the neuronal processes indicating MI-ER1 alpha expression.

3.1.3 Skeletal muscle

Cytoplasmic expression was detected at a high level with the pan antibody in embryonic myocytes (Figure 15 C and E), while expression was not detected with the alpha antibody (Figure 15 G and I), indicating that MI-ER1 beta is the only isoform expressed in embryonic skeletal muscle. In myocytes of adult skeletal muscle tissue, a low level of cytoplasmic expression was detected with both the pan and the alpha antibody (Figure 16). Therefore, MI-ER1 alpha is expressed only in the cytoplasm of cells in this tissue and it is possible that MI-ER1 beta is expressed in a similar manner.

3.1.4 Heart

Strong cytoplasmic expression was detected in the embryonic endocardial heart cells of the atria with pan anti-MI-ER1 (Figure 17 C and E), while expression was not detected with the alpha antibody (Figure 17 G and I), indicating that only the beta isoform is expressed in the embryonic heart cells. Because one of mouse embryo sections from Novagen did not contain any part of the embryonic ventricle of the heart, it was impossible to ascertain the level of staining in this part of the tissue. As a result of this, only the cells of the embryonic atrium could be analyzed with the pan antibody. In myocardiocytes of the adult heart, both the pan and the alpha antibody detected a moderate level of expression of MI-ER1 in the cytoplasm, (Figure 18) therefore, we can conclude that only MI-ER1 alpha is expressed.

3.1.5 Lung

In the embryonic lung, a strong level of cytoplasmic staining was detected with the pan antibody (Figure 19 C and E) in stromal cells. In the epithelium cells of the bronchi, only the cytoplasm was stained. No expression was detected with anti MI-ER1 alpha (Figure 19 G and I). This shows that MI-ER1 beta is the only isoform expressed in cells of the embryonic lung and this expression was both nuclear and cytoplasmic. In the adult lung, the staining intensity was significantly lower. In the bronchial epithelial cells, the cytoplasm was weakly stained with both the pan (Figure 20 C and D) and alpha (Figure 20 G and H) antibodies. This indicates that MI-ER1 alpha and possibly beta are expressed in the cell cytoplasm. No expression was detected in the alveolar epithelium with either antibody.

3.1.6 Digestive Tract

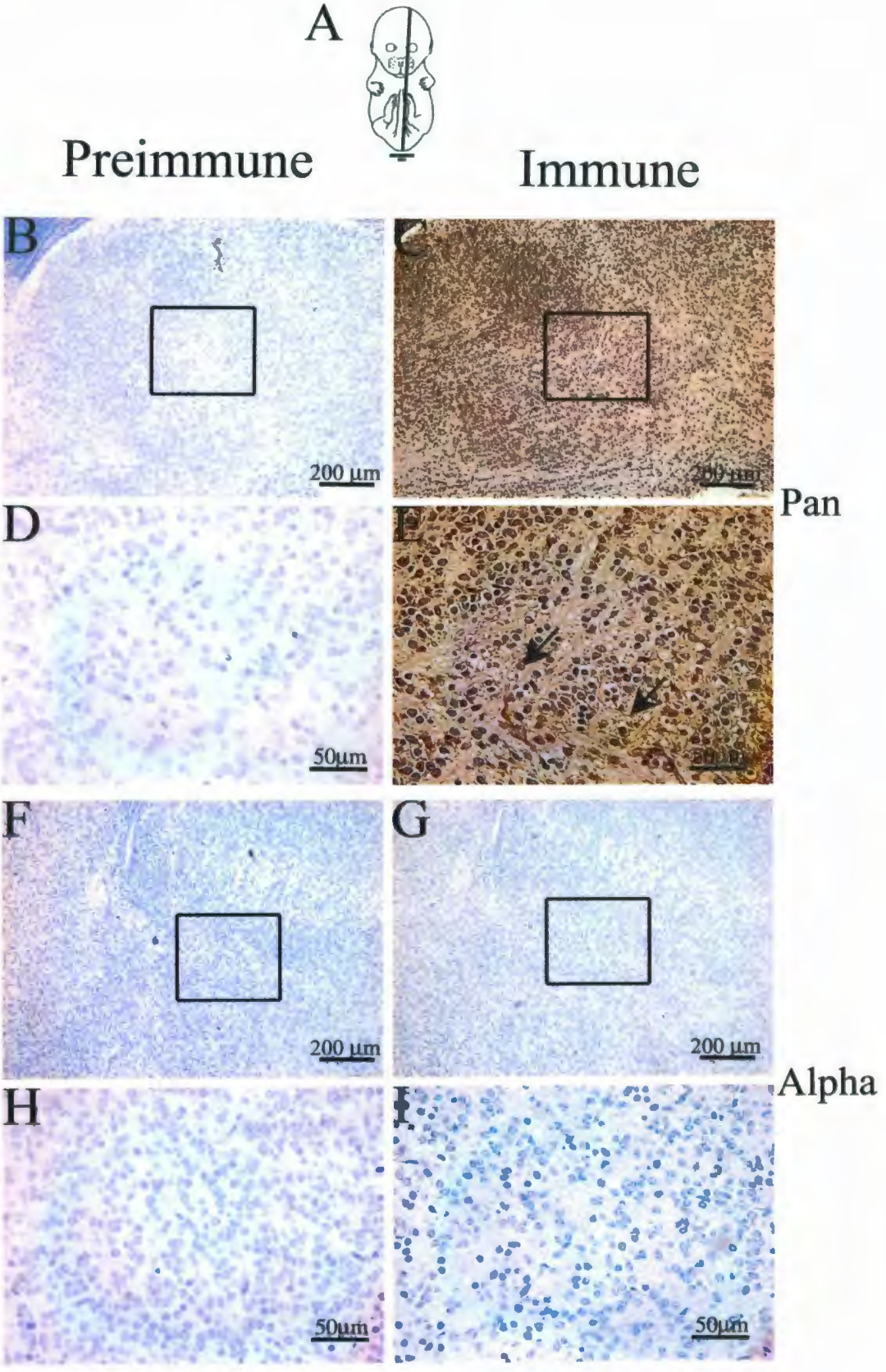
In the embryonic mouse digestive tract (Figure 21) strong cytoplasmic expression was found with the pan antibody in the enterocytes of the villi and smooth muscle cells. Expression was not detected with the anti-MI-ER1 alpha. Thus, MI-ER1 beta is expressed in the cytoplasm of these cells. In the enterocytes of the adult intestine, a moderate level of staining was observed for the pan antibody, while a weak level was detected with the alpha antibody (Figure 22). Thus, we can conclude that alpha is expressed in the cytoplasm of the enterocytes.

3.1.7 Liver

There are two types of cells in the embryonic liver: the hepatocytes and the hematopoietic cells. In the 16-day mouse embryonic liver, there was one cell type where strong expression was detected with the pan antibody (Figure 23 C and E) in the nucleus and cytoplasm; the other cell type did not express MI-ER1. These two cell types can be differentiated according to their morphology (one cell type was large and irregular, while the other cell was smaller and more rounded in shape), however, specific differentiation of the different cell types could not be determined. No expression was detected with the alpha antibody (Figure 23 G and I), indicating that MI-ER1 beta is expressed in the nucleus and cytoplasm of embryonic liver cells. In the adult liver (Figure 24), the pan antibody detected a moderate level of expression in the nuclei and the cytoplasm of the hepatocytes, while only the cytoplasm was weakly stained with anti- MI-ER1 alpha. We can therefore conclude that alpha is expressed in the cytoplasm, while beta is expressed in the nucleus and possibly cytoplasm of adult mouse liver hepatocytes.

Figure 10. Expression of MI-ER1 in the embryonic mouse brain.

The estimated position of the sections taken from the 16-day old mouse embryo for subsequent immunohistochemistry is indicated (A). Hindbrain of the 16 day mouse embryo was stained with preimmune sera (B,D) or pan anti-MI-ER1 (C,E) and preimmune sera (F,H) or anti-MI-ER1 alpha (G,I). Boxes shown in B and C correspond to panels D and E while boxes in F and G correspond to panels H and I, respectively. Arrows indicate MI-ER1 expression in cell nuclei and cell cytoplasm. Immunostaining appears brown while counterstain appears blue.


A
Preimmune
Immune
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C
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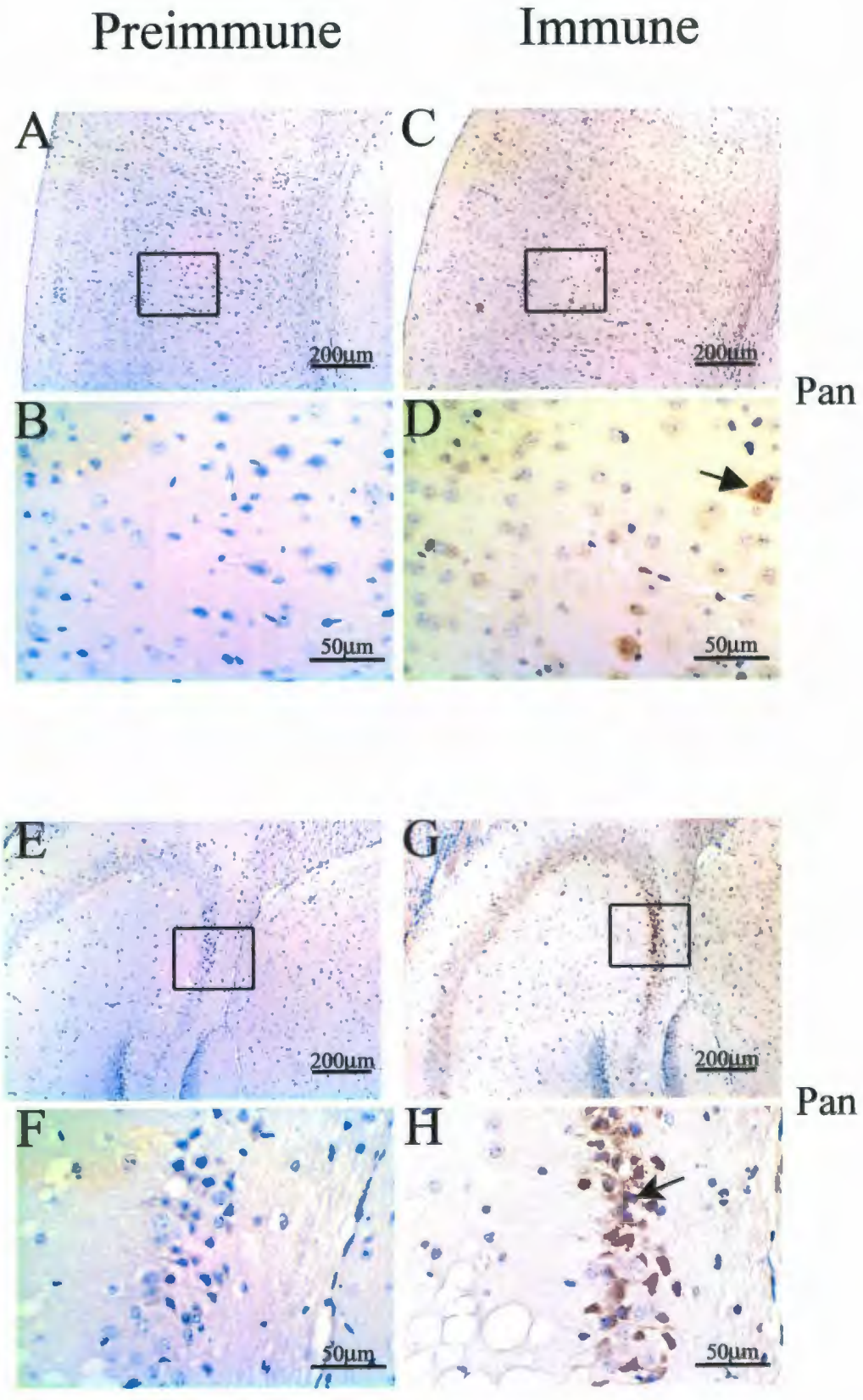
Pan
D
50µm
E
50µm
F
200 µm
G
200 µm
Alpha
H
50µm
I
50µm

Figure 11. Expression of MI-ER1 in the cerebral cortex and hippocampus of the adult mouse brain.

The cerebral cortex (A-D) and the hippocampus (E-H) of the adult mouse brain were stained with preimmune sera (A,B and E,F) or pan anti-MI-ER1 (C,D and G,H). Boxes shown in A and C correspond to panels B and D while boxes in E and G correspond to panels F and G, respectively. Arrows indicate staining in the nuclei and cytoplasm of pyramidal cells (D) and the cytoplasm of CA3 cells (H). Immunostaining appears brown while counterstain appears blue.


Preimmune
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A
C
200µm
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Pan
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50µm
50µm
E
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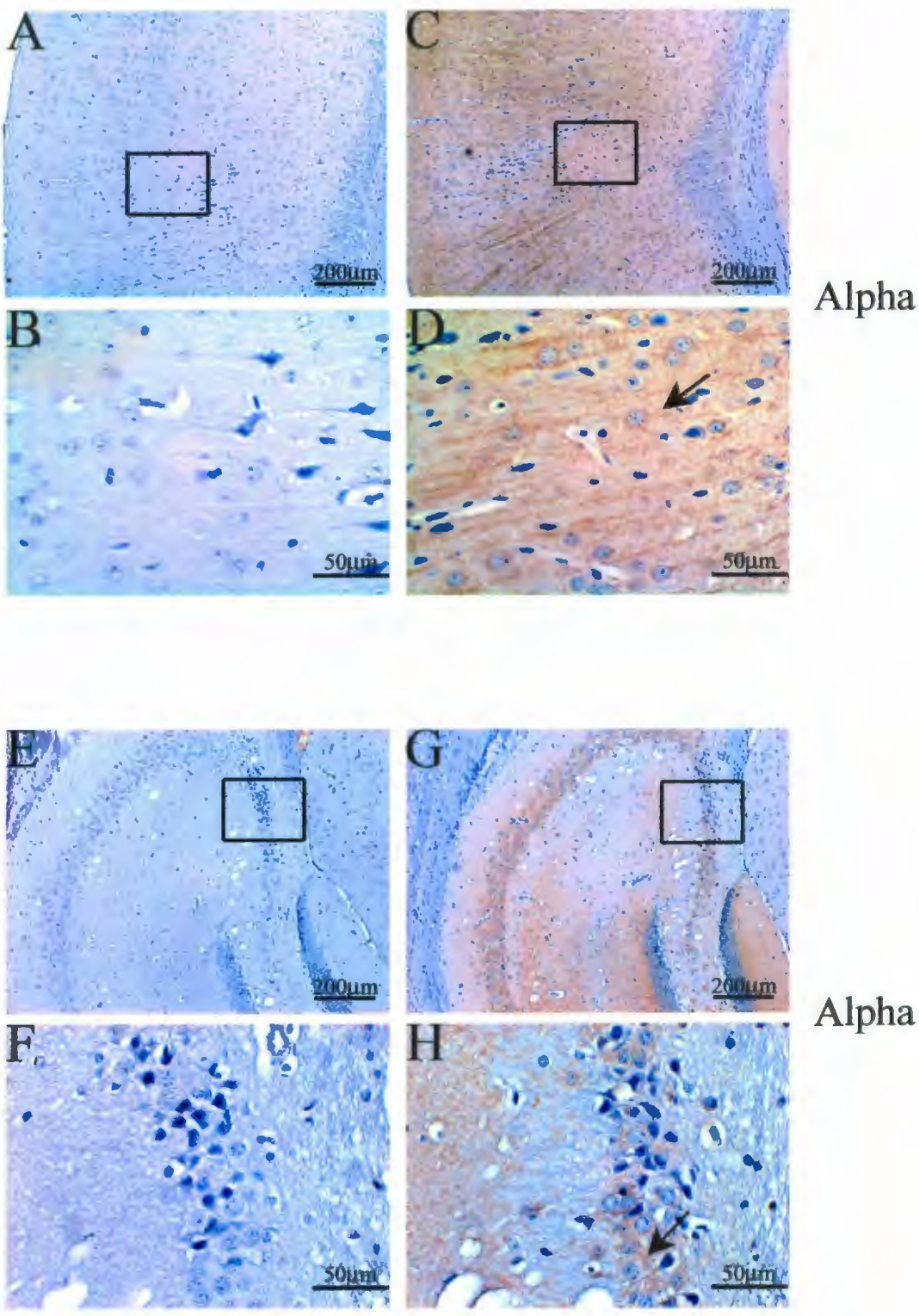
200µm
200µm
Pan
F
H
50µm
50µm

Figure 12. Expression of MI-ER1 alpha in the cerebral cortex and hippocampus of the adult mouse brain.

The cerbral cortex (A-D) and the hippocampus (E-H) of the adult mouse brain were stained with preimmune sera (A,B and E,F) or anti-MI-ER1 alpha (C,D and G,H). Boxes shown in A and C correspond to panels B and D while boxes in E and G correspond to panels F and H, respectively. Arrows indicate staining in the cytoplasm of the pyramidal cells (D) and CA3 cells (H). Immunostaining appears brown while counterstain appears blue.


Preimmune
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Alpha
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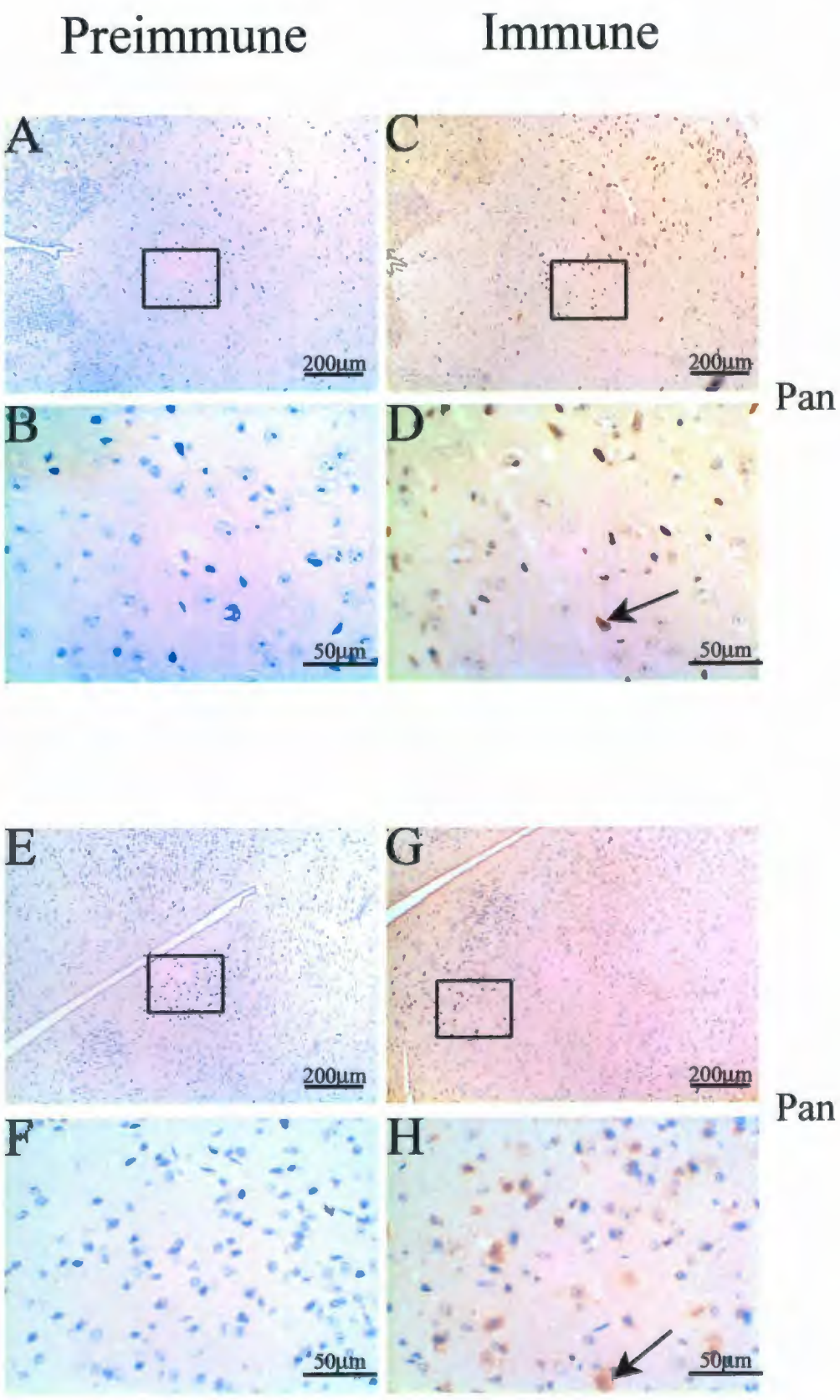
50µm
50µm

Figure 13. Expression of MI-ER1 in the thalamus and hypothalamus of the adult mouse brain.

The thalamus (A-D) and the hypothalamus (E-H) of the adult mouse brain were stained with preimmune sera (A,B and E,F) or pan anti-MI-ER1 (C,D and G,H). Boxes shown in A and C correspond to panels B and D while boxes in E and G correspond to panels F and H, respectively. Arrows indicate staining in the pyramidal cells (D) and neurons (H). Immunostaining appears brown while counterstain appears blue.


Preimmune
Immune
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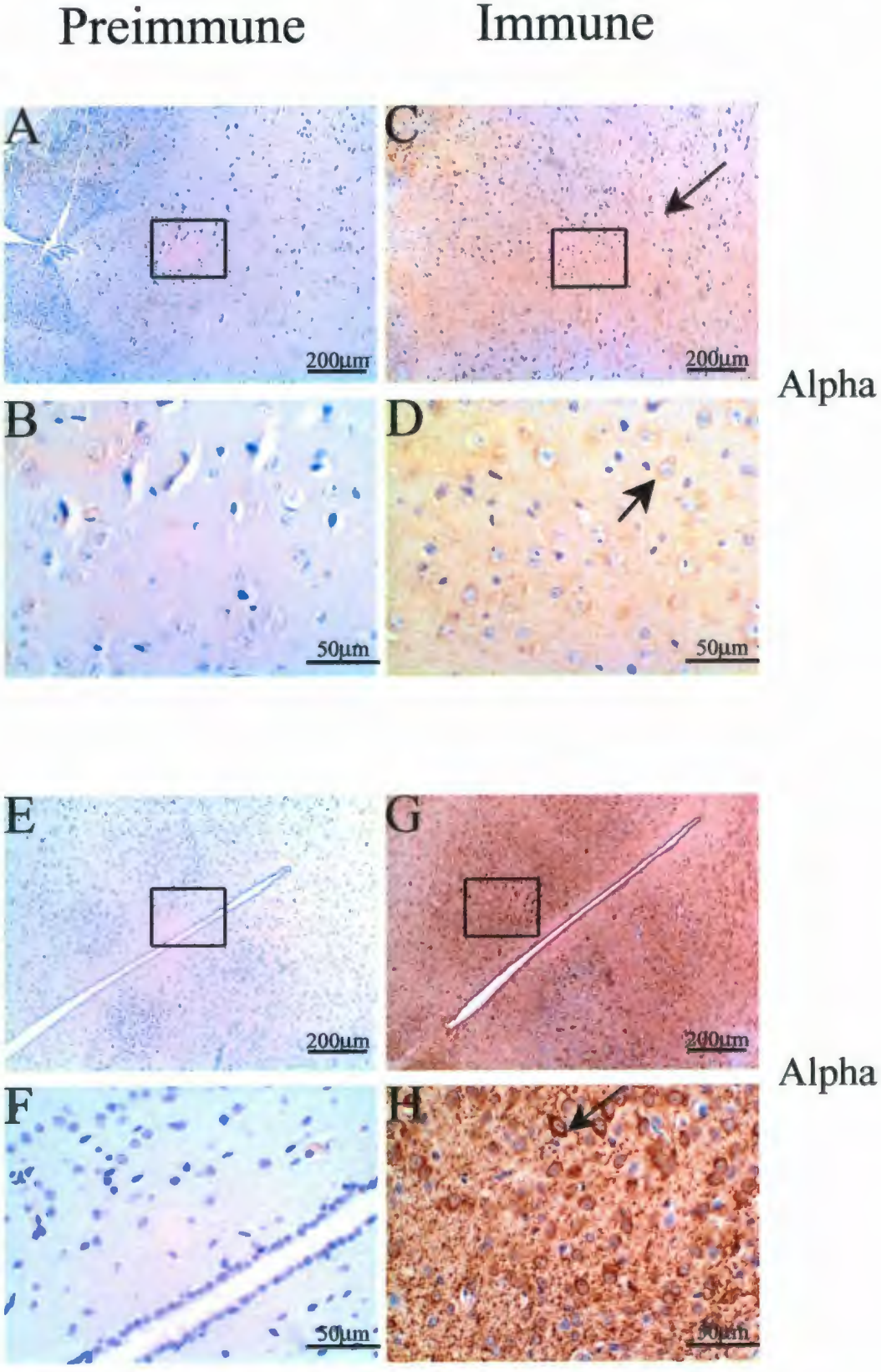
C
200μm
200μm
Pan
B
D
50μm
50μm
E
G
200μm
200μm
Pan
F
H
50μm
50μm

Figure 14. Expression of MI-ER1 alpha in the thalamus and hypothalamus of the adult mouse brain.

The thalamus (A-D) and the hypothalamus (E-H) of the adult mouse brain were stained with preimmune sera (A,B and E,F) or pan anti-MI-ER1 (C,D and G,H). Boxes shown in A and C correspond to panels B and D while boxes in E and G correspond to panels F and H, respectively. Arrows indicate staining in the cytoplasm of the pyramidal cells (D) and neurons (H). Immunostaining appears brown while counterstain appears blue.


Preimmune
Immune
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C
200µm
200µm
Alpha
B
D
50µm
50µm
E
G
200µm
200µm
Alpha
F
H
50µm
50µm

Figure 15. Expression of MI-ER1 in embryonic mouse skeletal muscle.

The estimated position of the sections taken from the 16 day old mouse embryo for subsequent immunohistochemistry is indicated (A). Skeletal muscle of the 16 day mouse embryo was immunostained with preimmune sera (B,D) or pan anti-MI-ER1 (C,E) and preimmune sera (F,H) or anti-MI-ER1 alpha (G,I). Boxes shown in B and C correspond to panels D and E while boxes in F and G correspond to panels H and I, respectively. Arrows indicate MI-ER1 expression in cell nuclei and cell cytoplasm. Immunostaining appears brown while counterstain appears blue.

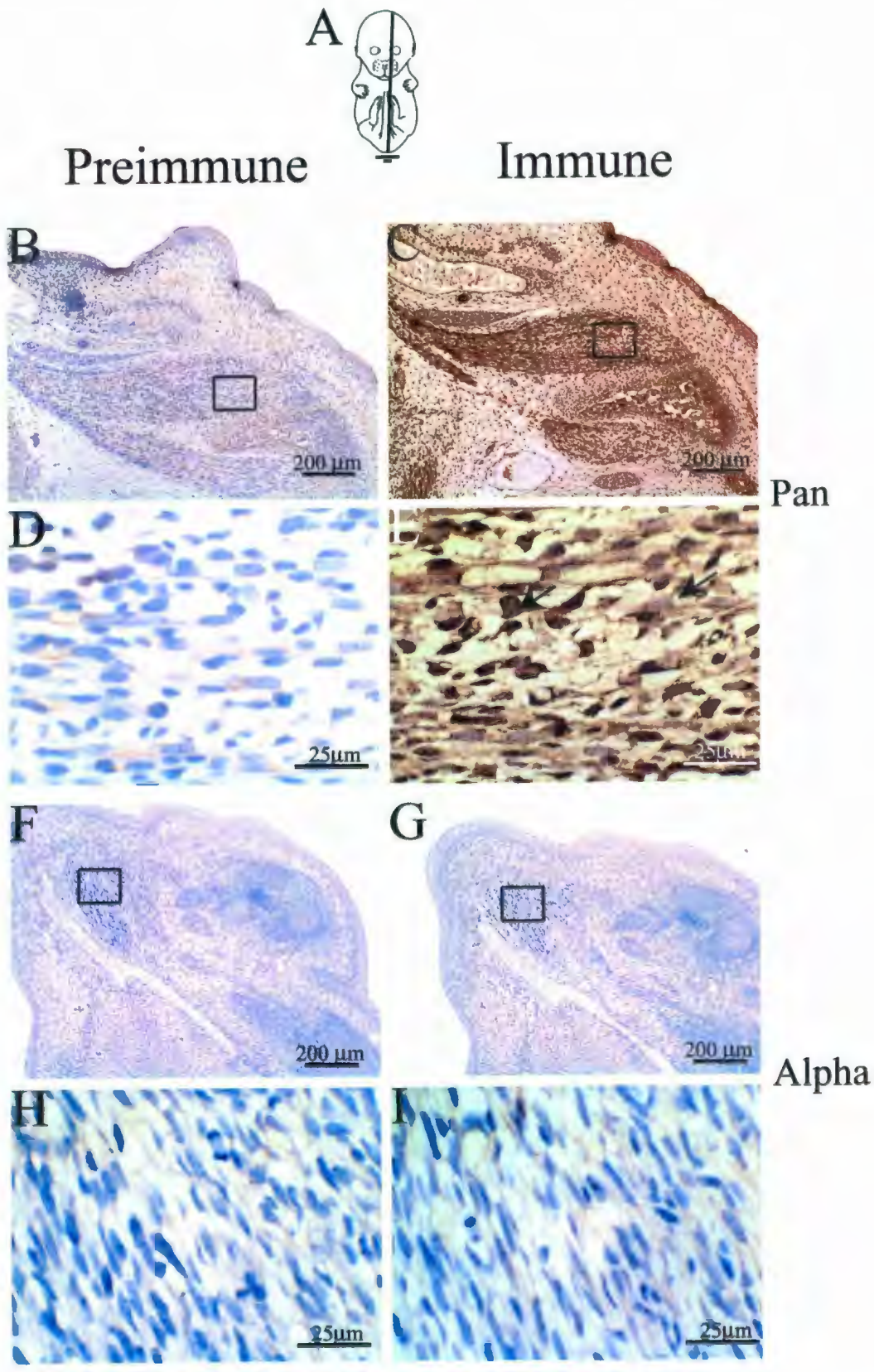
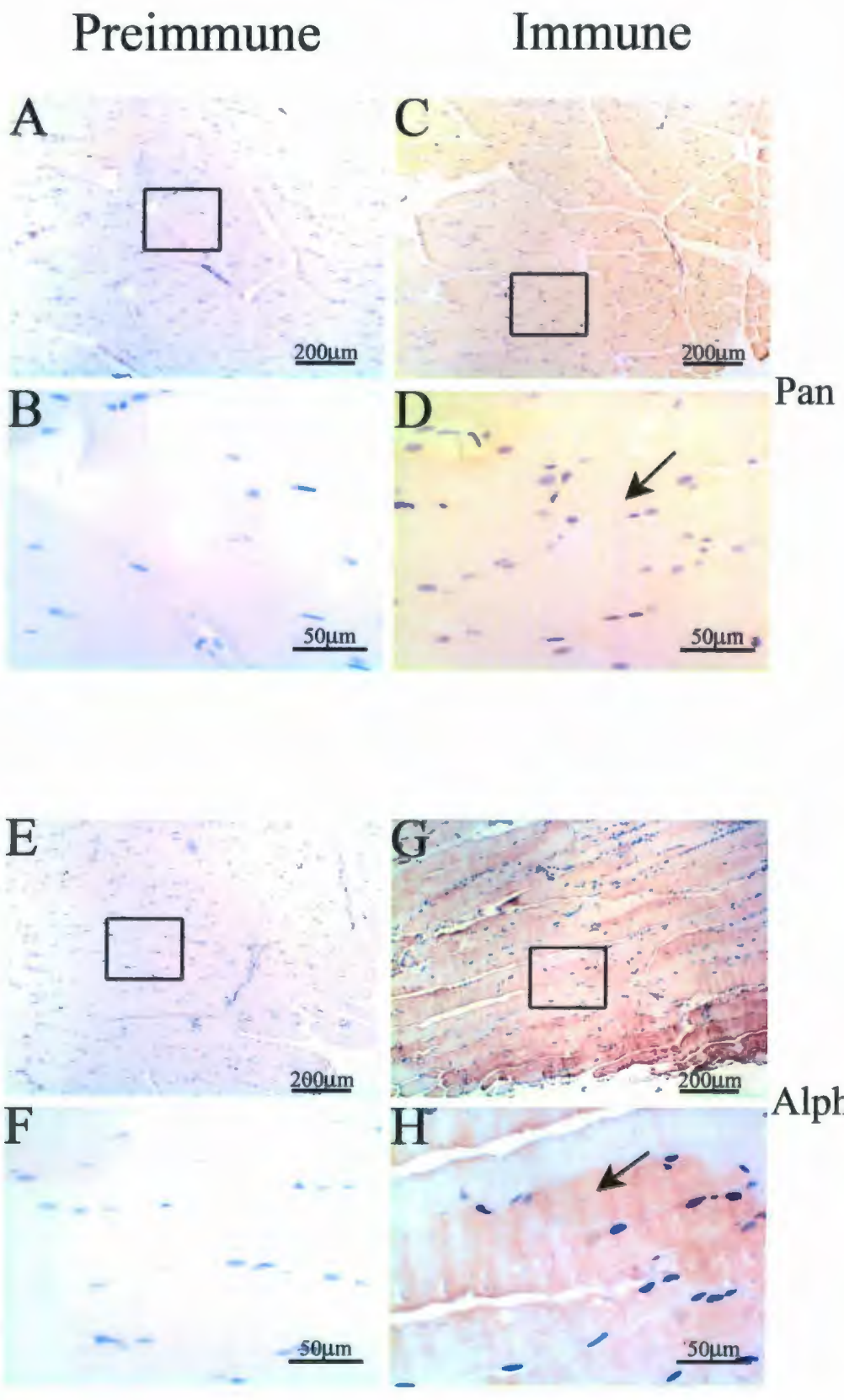

A
Preimmune
Immune
B
C
200 µm
200 µm
Pan
D
25µm
F
G
200 µm
200 µm
Alpha
H
I
25µm
25µm

Figure 16. Expression of MI-ER1 in adult mouse skeletal muscle.

Skeletal muscle of the adult mouse was stained with preimmune sera (A,B) or pan anti-MI-ER1 (C,D) and preimmune sera (E,F) and anti-MI-ER1 alpha (G,H). Boxes shown in A and C correspond to panels B and D and boxes shown in E and G correspond to panels F and H respectively. Arrows indicate cytoplasmic staining in myocytes. Immunostaining appears brown while counterstaining appears blue.


Preimmune
Immune
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C
200µm
200µm
Pan
B
D
50µm
50µm
E
G
200µm
200µm
Alpha
F
H
50µm
50µm

Figure 17. Expression of MI-ER1 in the embryonic mouse heart.

The estimated position of the sections taken from the 16 day old mouse embryo for subsequent immunohistochemistry is indicated (A). Heart of the 16 day mouse embryo was immunostained with preimmune sera (B,D) or pan anti-MI-ER1 (C,E) and preimmune sera (F,H) or anti-MI-ER1 alpha (G,I). Boxes shown in B and C correspond to panels D and E while boxes in F and G correspond to panels H and I, respectively. Arrows indicate MI-ER1 expression in cell cytoplasm. Immunostaining appears brown while counterstain appears blue.

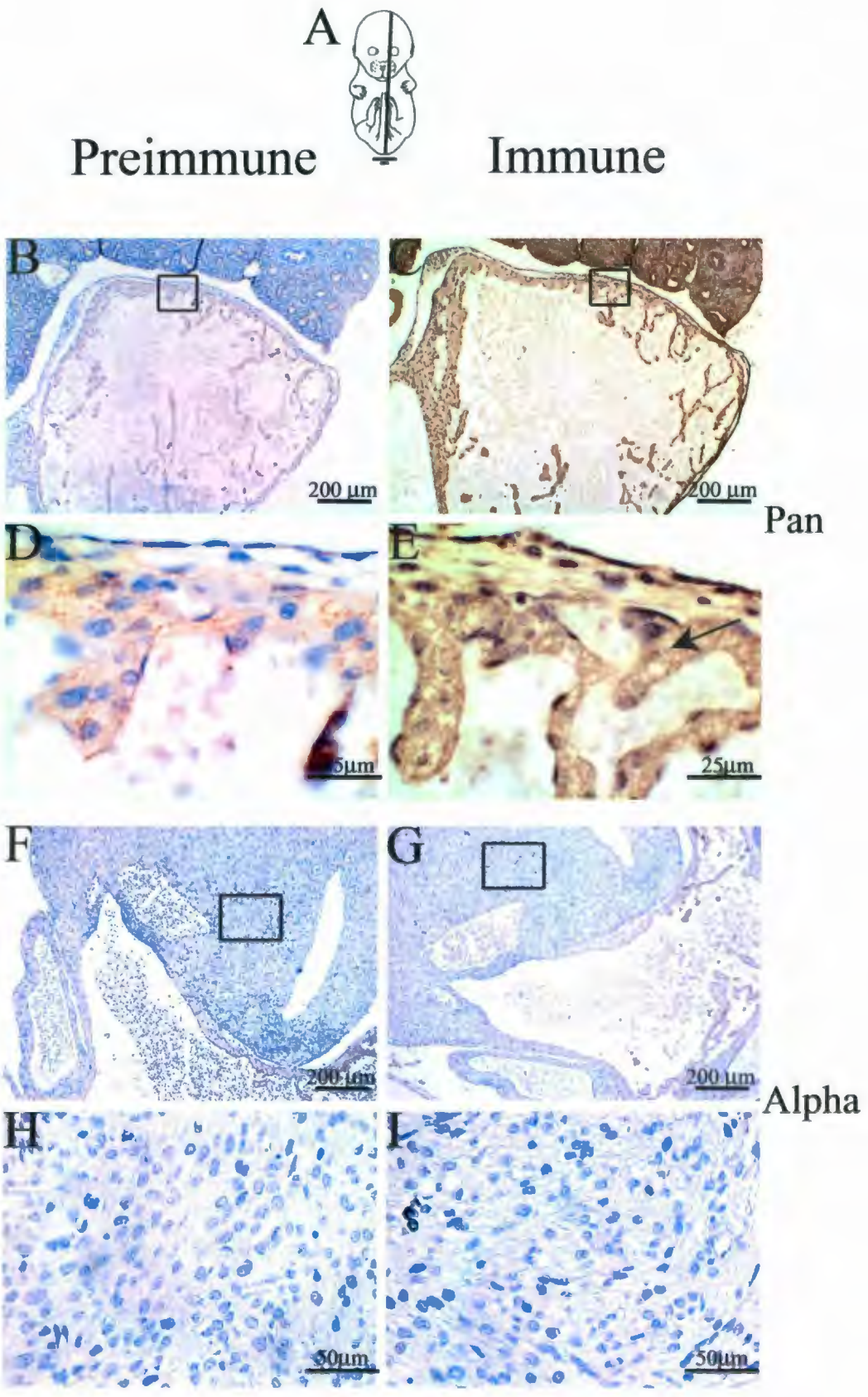

A
Preimmune
Immune
B
C
200 µm
200 µm
Pan
D
E
5µm
25µm
F
G
200 µm
200 µm
Alpha
H
I
50µm
50µm

Figure 18. Expression of MI-ER1 in the adult mouse heart.

The heart ventricle of the mouse was stained with preimmune sera (A,B) or pan anti-MI-ER1 (C,D) and preimmune sera (E,F) and anti-MI-ER1 alpha (G,H). Boxes shown in A and C correspond to panels B and D and boxes shown in E and G correspond to panels F and H respectively. Arrows indicate cytoplasmic staining in myocytes. Immunostaining appears brown while counterstaining appears blue.

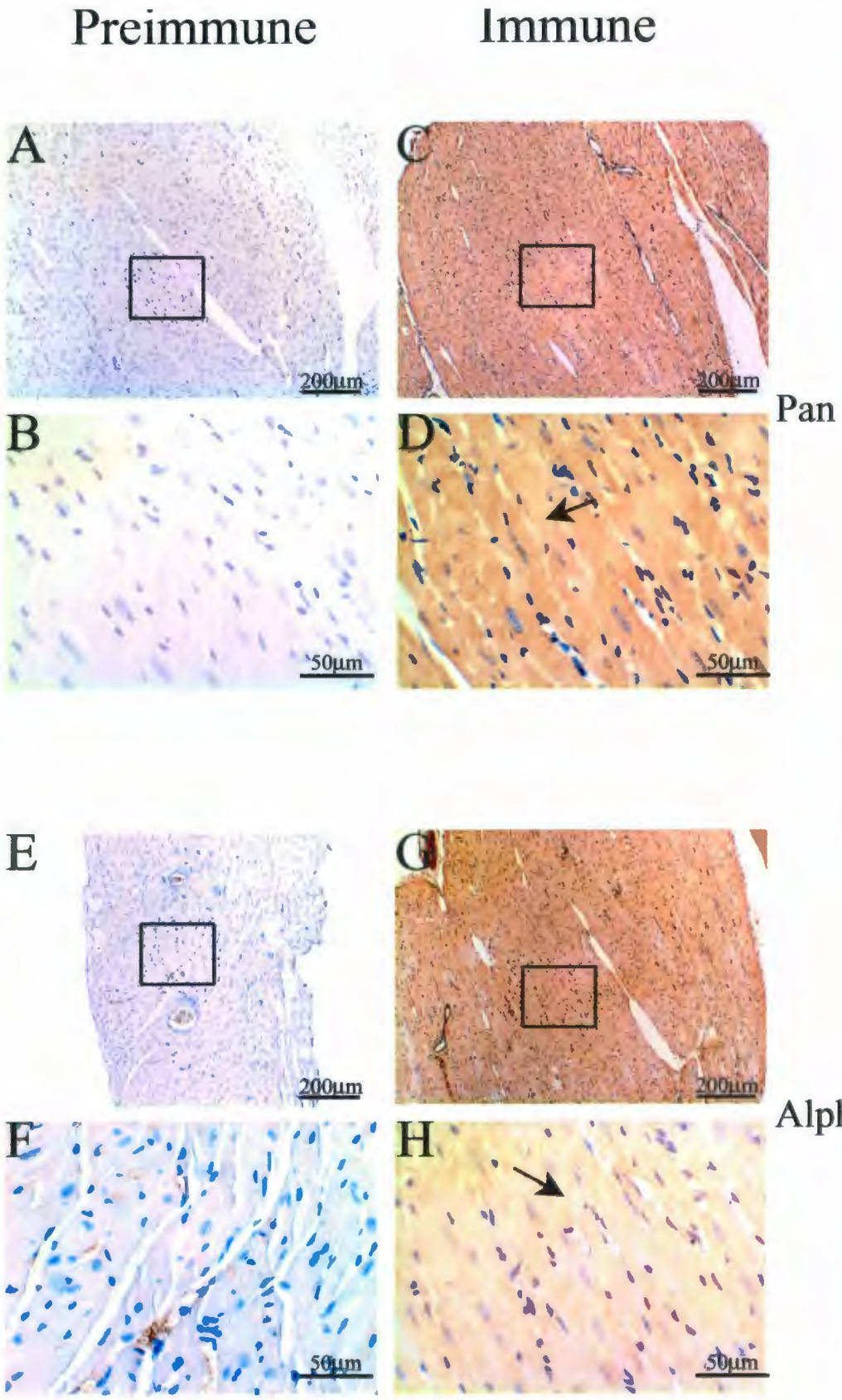

Preimmune
Immune
A
C
200µm
200µm
Pan
B
D
50µm
50µm
E
G
200µm
200µm
Alpha
F
H
50µm
50µm

Figure 19. Expression of MI-ER1 in the embryonic mouse lung.

The estimated position of the sections taken from the 16 day old mouse embryo for subsequent immunohistochemistry is indicated (A). Lung of the 16 day mouse embryo was immunostained with preimmune sera (B,D) or pan anti-MI-ER1 (C,E) and preimmune sera (F,H) or anti-MI-ER1 alpha (G,I). Boxes shown in B and C correspond to panels D and E while boxes in F and G correspond to panels H and I, respectively. Arrows indicate MI-ER1 expression in bronchial epithelial cell cytoplasm. Immunostaining appears brown while counterstain appears blue. Br=bronchus;St = stroma.

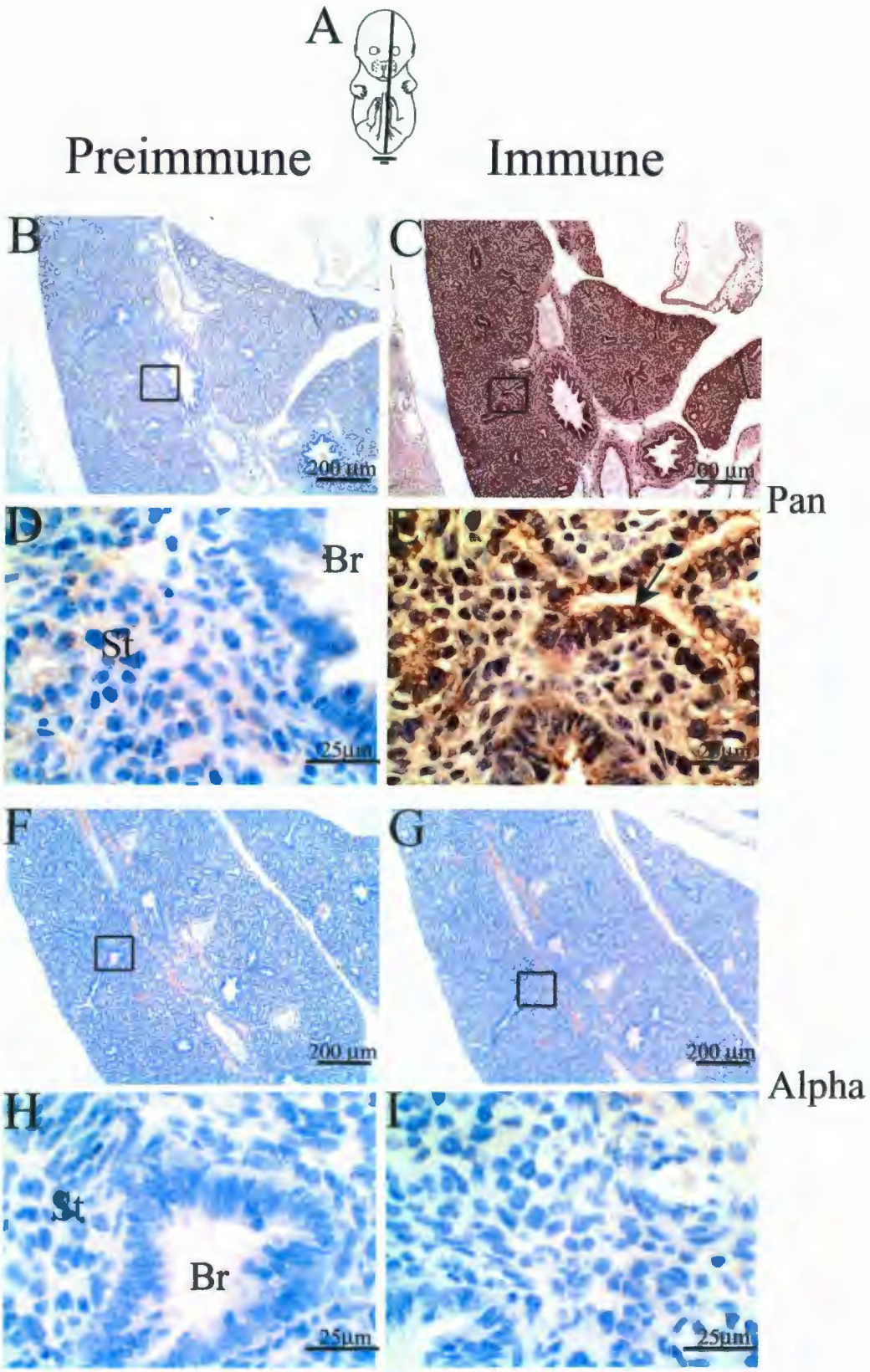

A
Preimmune
Immune
B
C
200 µm
200 µm
Pan
D
Br
St
25µm
25µm
F
G
200 µm
200 µm
Alpha
H
St
Br
25µm
I
25µm

Figure 20: Expression of MI-ER1 in the mouse adult lung.

The lung of the mouse was stained with preimmune sera (A,B) or pan anti-MI-ER1 (C,D) and preimmune sera (E,F) and anti-MI-ER1 alpha (G,H). Boxes shown in A and C correspond to panels B and D and boxes shown in E and G correspond to panels F and H respectively. Arrows indicate cytoplasmic staining in bronchial and alveolar epithelium. Immunostaining appears brown while counterstaining appears blue. EP=epithelium;AV= alveoli.

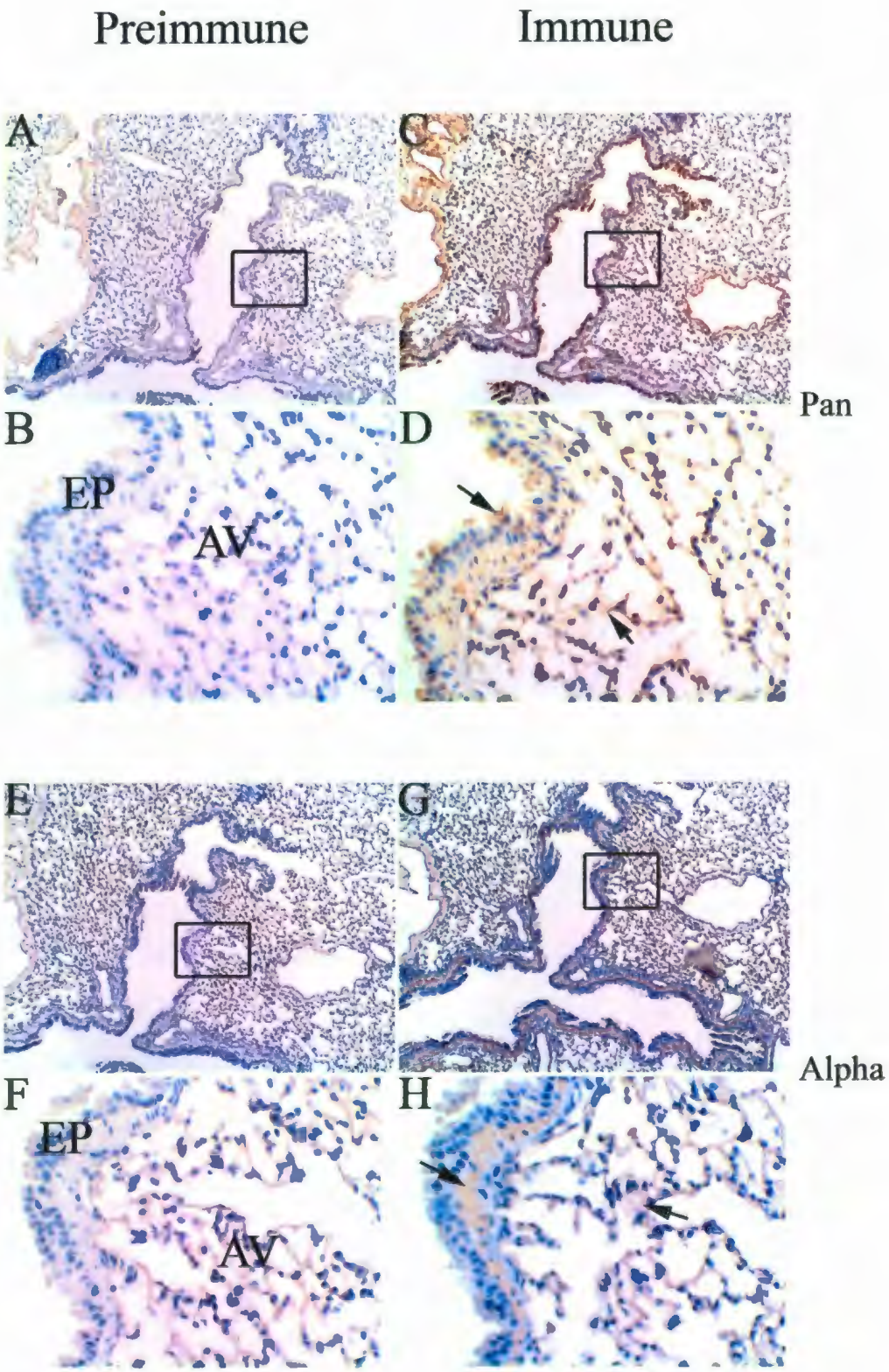

Preimmune
Immune
A
C
B
D
EP
AV
Pan
E
G
F
H
EP
AV
Alpha

Figure 21. Expression of MI-ER1 in the embryonic mouse digestive tract.

The estimated position of the sections taken from the 16 day old mouse embryo for subsequent immunohistochemistry is indicated (A). The digestive tract of the 16 day mouse embryo was immunostained with preimmune sera (B,D) or pan anti-MI-ER1 (C,E) and preimmune sera (F,H) or anti-MI-ER1 alpha (G,I). Boxes shown in B and C correspond to panels D and E while boxes in F and G correspond to panels H and I, respectively. Arrows indicate MI-ER1 expression in cell nuclei and cell cytoplasm. Immunostaining appears brown while counterstain appears blue. V = villi;LP = lamina propria.

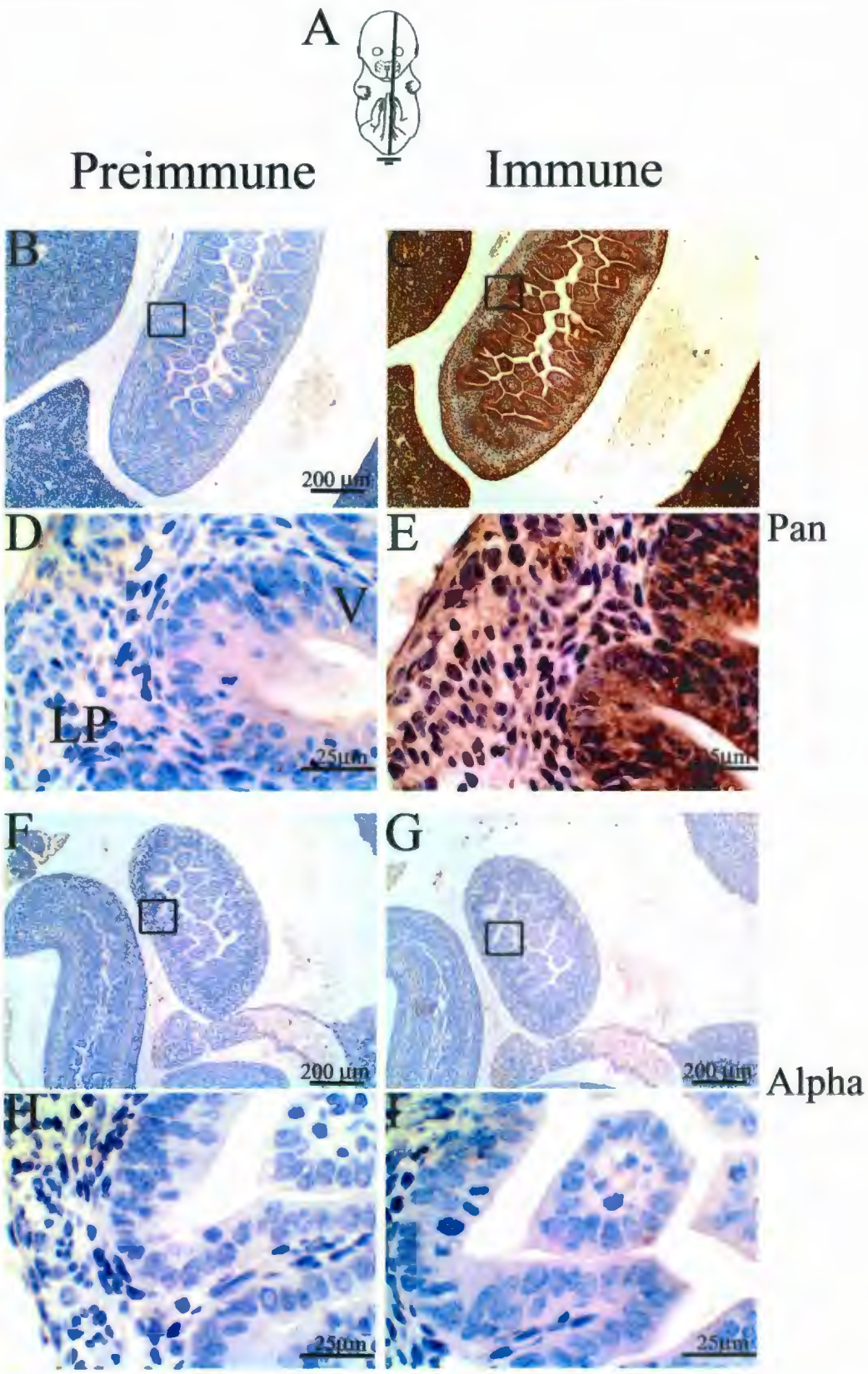

A
Preimmune
Immune
B
200 µm
D
V
LP
25µm
E
Pan
F
200 µm
G
200 µm
Alpha
H
25µm
25µm

Figure 22: Expression of MI-ER1 in the adult mouse digestive tract.

The digestive tract of the adult mouse was stained with preimmune sera (A,B) or pan anti-MI-ER1 (C,D) and preimmune sera (E,F) and anti-MI-ER1 alpha (G,H). Boxes shown in A and C correspond to panels B and D and boxes shown in E and G correspond to panels F and H respectively. Arrows indicate cytoplasmic staining cells of the villi. Immunostaining appears brown while counterstaining appears blue.

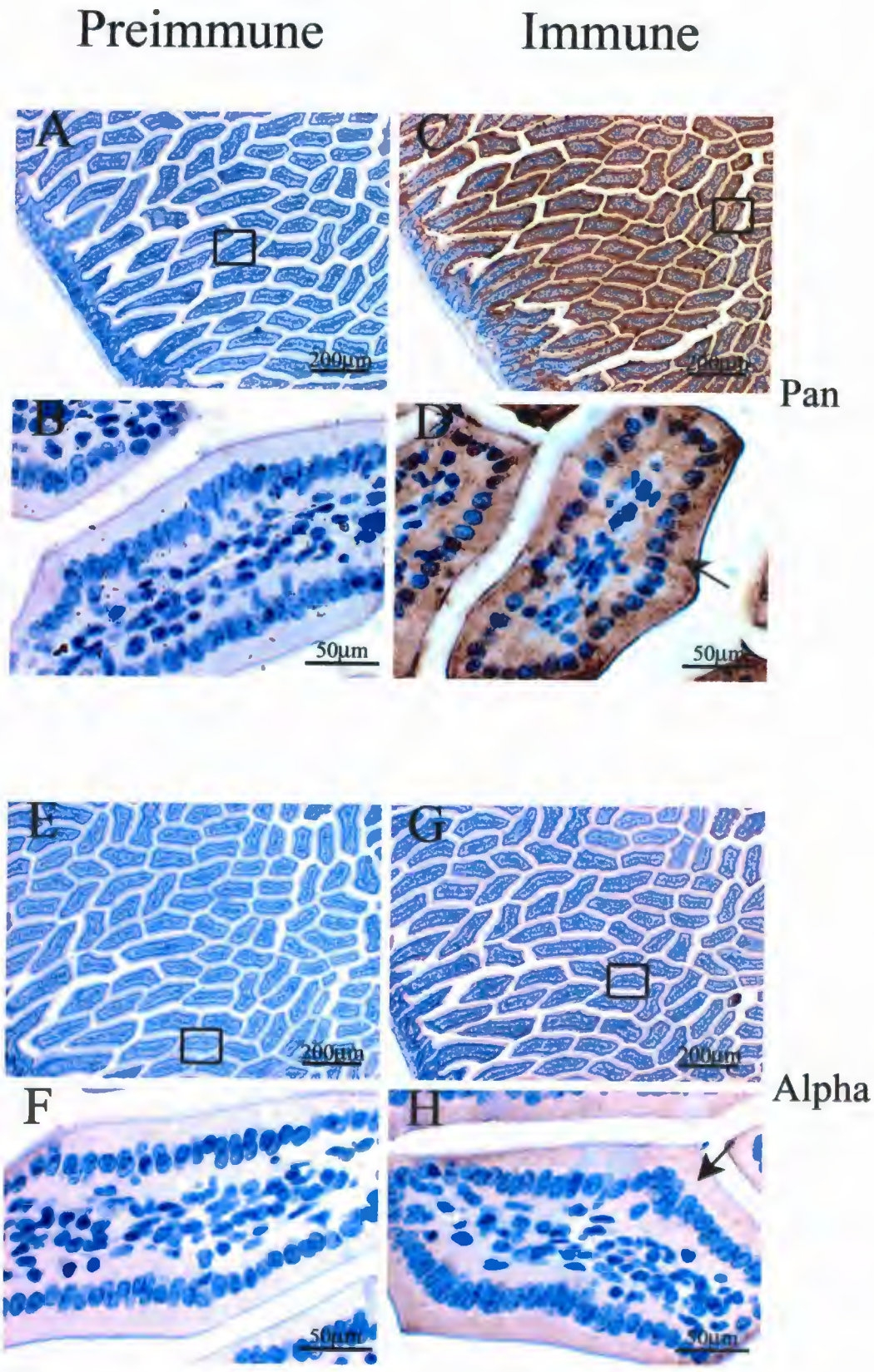

Preimmune
Immune
A
C
200μm
200μm
Pan
B
D
50μm
50μm
E
G
200μm
200μm
Alpha
F
H
50μm
50μm

Figure 23. Expression of MI-ER1 in the embryonic mouse liver.

The estimated position of the sections taken from the 16 day old mouse embryo for subsequent immunohistochemistry is indicated (A). The liver of the 16 day mouse embryo was immunostained with preimmune sera (B,D) or pan anti-MI-ER1 (C,E) and preimmune sera (F,H) or anti-MI-ER1 alpha (G,I). Boxes shown in B and C correspond to panels D and E while boxes in F and G correspond to panels H and I, respectively. Arrows the different cell types in the embryonic liver (E). Immunostaining appears brown while counterstain appears blue.

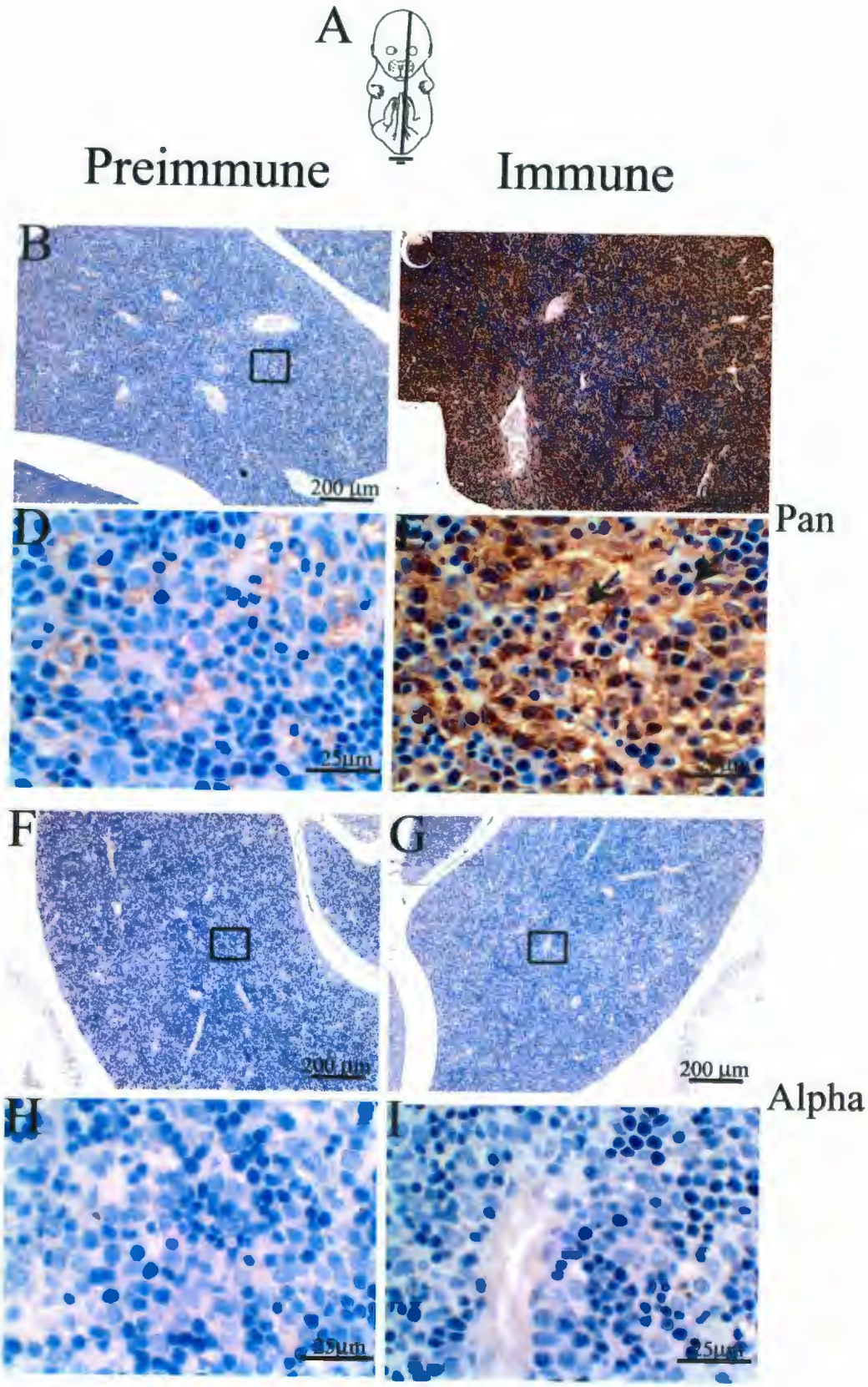
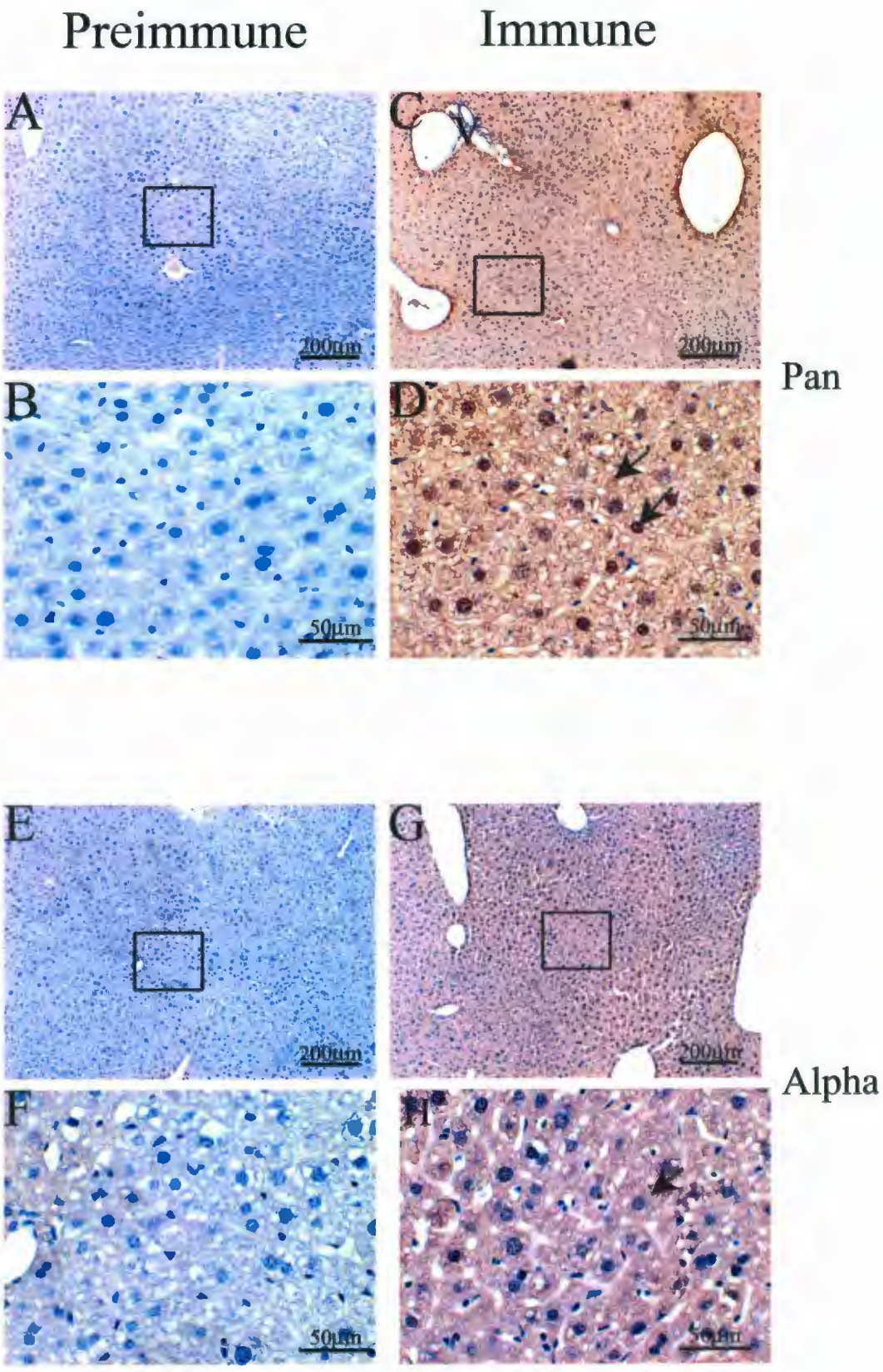

A
Preimmune
Immune
B
C
200 µm
D
E
25µm
Pan
F
G
200 µm
200 µm
H
I
25µm
25µm
Alpha

Figure 24. Expression of MI-ER1 in the adult mouse liver.

The liver of the adult mouse was stained with preimmune sera (A,B) or pan anti-MI-ER1 (C,D) and preimmune sera (E,F) and anti-MI-ER1 alpha (G,H). Boxes shown in A and C correspond to panels B and D and boxes shown in E and G correspond to panels F and H respectively. Arrows indicate cytoplasmic (D,H) and nuclear (D) staining in the hepatocytes. Immunostaining appears brown while counterstaining appears blue. V=vessel.


Preimmune
Immune
A
C
200μm
200μm
B
D
50μm
50μm
Pan
E
G
200μm
200μm
F
H
50μm
50μm
Alpha

3.2 Expression of MI-ER1 alpha and beta in other adult mouse tissues

The expression pattern of MI-ER1 in other adult mouse tissues, those that did not have visible corresponding tissues in the 16-day embryo section, was examined. These tissues included the kidney and the spleen.

3.2.1 Kidney

In the adult kidney, a moderate level of nuclear and cytoplasmic expression was evident in all the epithelial cells of the cortical tubules when stained with the pan-antibody (Figure 25 C and D). Only cytoplasmic expression was evident with the alpha antibody in these cells (Figure 26 C and D) and this staining was less intense than that of the pan antibody. The most intense level of staining was seen in the cells of the outer cortex and in the cells of the medullary rays extending into the medulla. Overall, we can conclude that MI-ER1 beta is expressed in the nucleus and MI-ER1 alpha is expressed in the cytoplasm in the cells of the cortex and medulla. Interestingly, MI-ER1 expression was not detected with either antibody in the podocytes of the glomeruli. In the epithelial cells of medullary collecting tubules, both the nuclei and cytoplasm were moderately stained with the pan antibody (Figure 25 G and H), while only the cytoplasm was stained with the alpha antibody (moderate level of staining) (Figure 26 G and H), indicating that the beta isoform is expressed in the nucleus (possibly in the cytoplasm) and the alpha isoform is expressed in the cytoplasm of these epithelial cells.

3.2.2 Spleen

In the spleen, a moderate level of expression of MI-ER1 was detected with the pan antibody in the cytoplasm of the cells of the white pulp and weak nuclear and cytoplasmic expression in the cells of the red pulp (Figure 27). No expression was detected with the alpha antibody (Figure 28). Thus, only the beta isoform is expressed in the spleen and this expression is both nuclear and cytoplasmic.

Figure 25. Expression of MI-ER1 in the adult mouse kidney.

The cortex (A-D) and the medullla (E-H) of the adult mouse kidney were stained with preimmune sera (A,B and E,F) or pan anti-MI-ER1(C,D and G,H). Boxes shown in A and C correspond to panels B and D while boxes in E and G correspond to panels F and H, respectively. Arrows indicate nuclear and cytoplasmic staining in the epithelial cells of the cortical tubules (D) collecting tubules (H). Immunostaining appears brown while counterstain appears blue. CL = cortical labyrinth; CT = collecting tubule; G=glomerulus; MR = medullary rays.

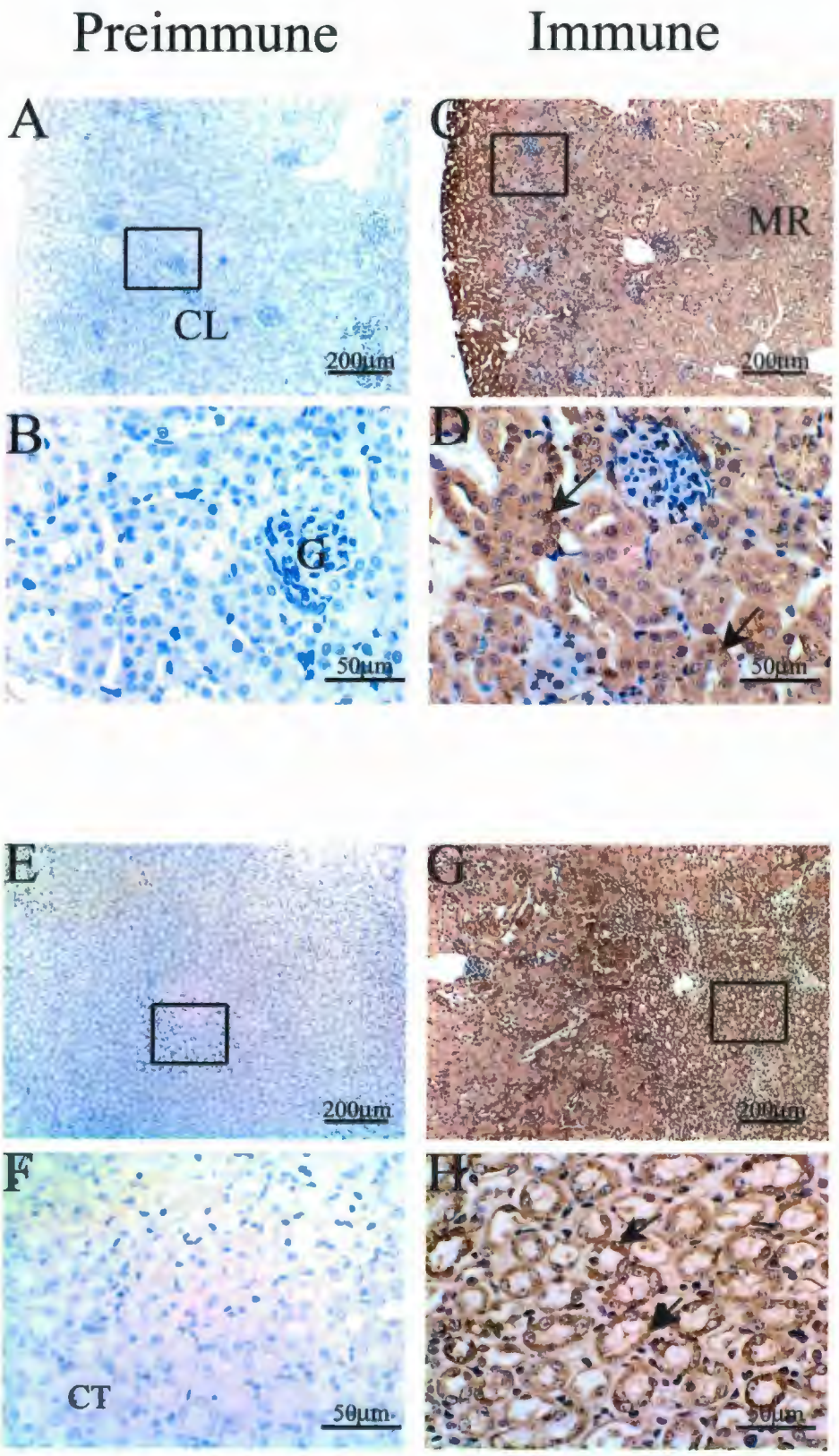

Preimmune
Immune
A
CL
200μm
C
MR
200μm
B
G
50μm
D
50μm
E
200μm
G
200μm
F
CT
50μm
H
50μm

Figure 26. Expression of MI-ER1 alpha in the adult mouse kidney.

The cortex (A-D) and the medullla (E-H) of the adult mouse kidney were stained with preimmune sera (A,B and E,F) or anti-MI-ER1 alpha (C,D and G,H). Boxes shown in A and C correspond to panels B and D while boxes in E and G correspond to panels F and H, respectively. Arrows indicate cytoplasmic staining in the epithelial cells of the cortical tubules (D) collecting tubules (H). Immunostaining appears brown while counterstain appears blue. CL = cortical labyrinth; CT = collecting tubule; G=glomerulus; MR = medullary rays.

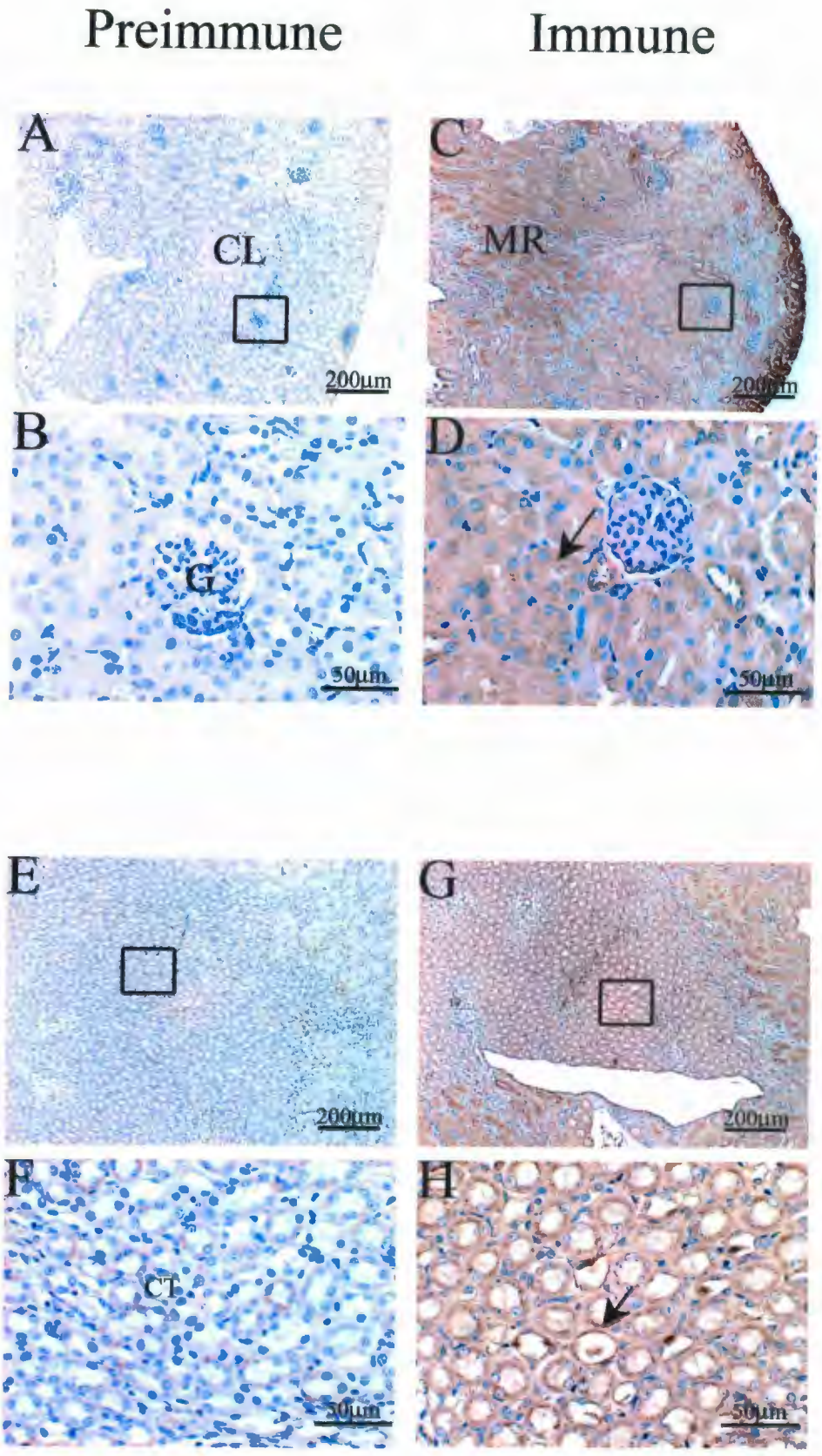

Preimmune
Immune
A
CL
200µm
C
MR
200µm
B
G
50µm
D
50µm
E
200µm
G
200µm
F
CT
50µm
H
50µm

Figure 27. Expression of MI-ER1 in the adult mouse spleen.

Mouse adult spleen was stained with preimmune sera (A,C,E) and pan anti-MI-ER1 (B,D,F). Boxes shown in A correspond to panels C and E while boxes shown in B correspond to panels D and F. Arrows indicate nuclear (F) and cytoplasmic (D and E) in cells of the spleen. Immunostaining appears brown while counterstain appears blue. WP= white pulp; RP = red pulp.

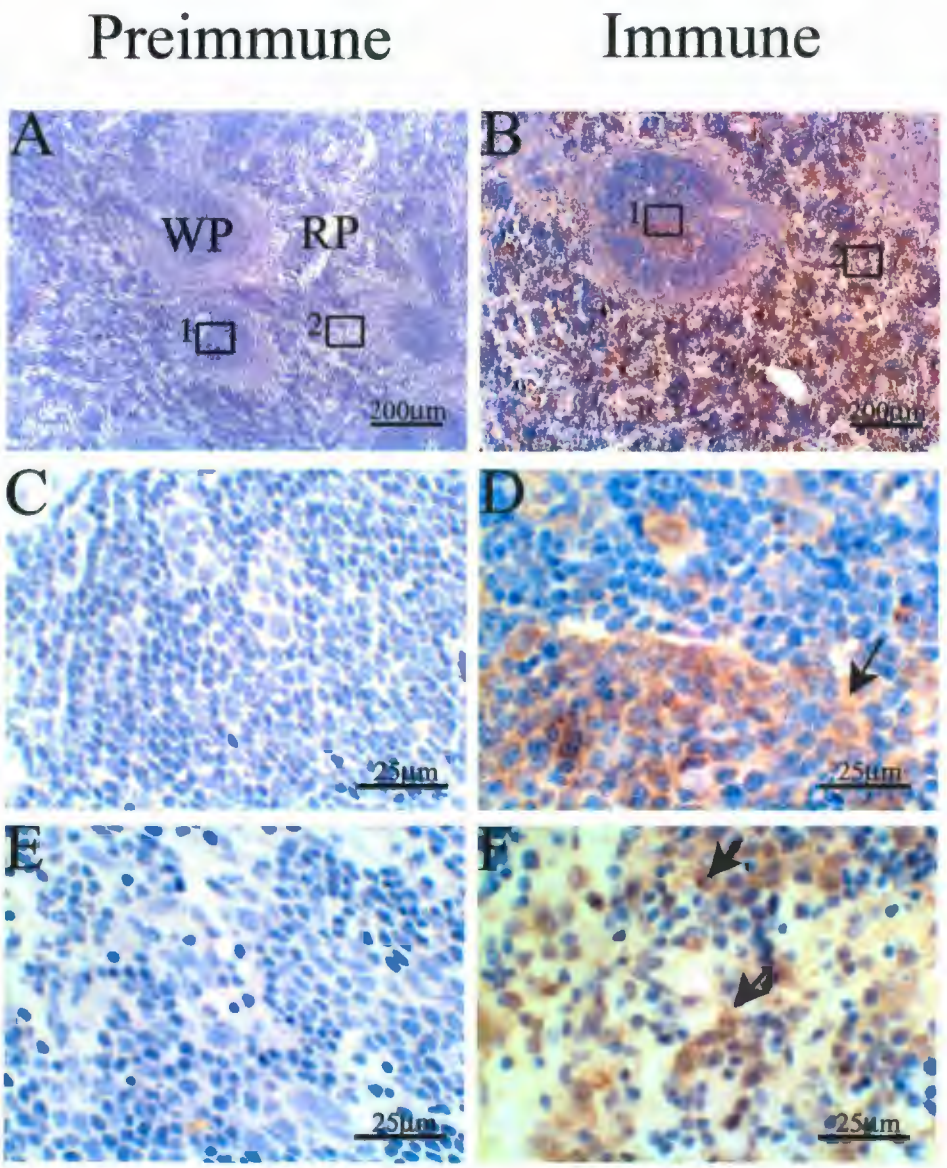
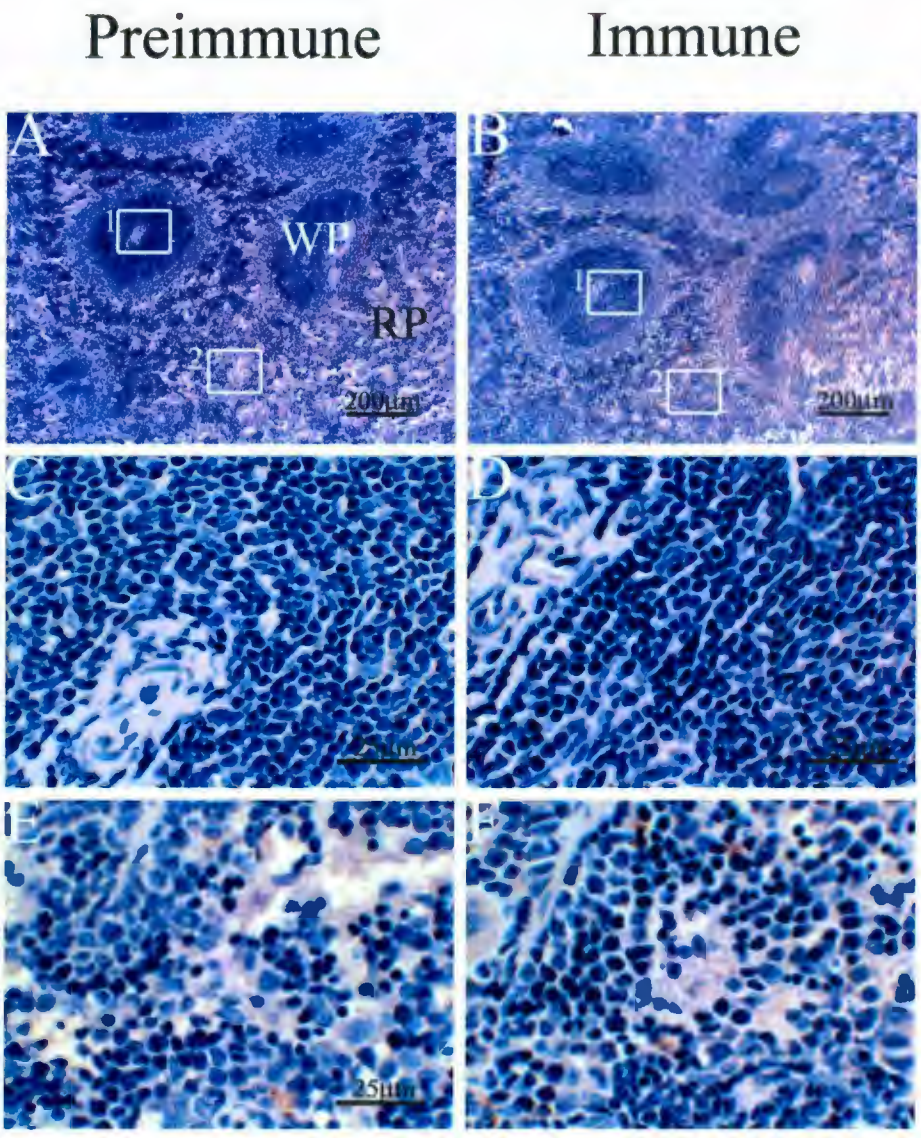

Preimmune
Immune
A
WP
RP
1
2
200μm
B
1
2
200μm
C
25μm
D
25μm
E
25μm
F
25μm

Figure 28: Expression of MI-ER1 alpha in the adult mouse spleen

Mouse adult spleen was stained with preimmune sera (A,C,E) and anti-MI-ER1 alpha (B,D,F). Boxes shown in A correspond to panels C and E while boxes shown in B correspond to panels D and F. Immunostaining appears brown while counterstain appears blue. WP= white pulp; RP = red pulp.


Preimmune
Immune
A
B
WP
RP
1
2
200μm
200μm
C
D
25μm
E
F
25μm

3.3 Expression of MI-ER1 in adult mouse endocrine and endocrine responsive tissues

Since MI-ER1 interacts with several nuclear steroid hormone receptors, including the estrogen receptor (Savicky *et al.* 2004), we investigated the expression pattern in various mouse endocrine organs and in organs that are responsive to estrogen, such as the adrenal gland, thyroid, pancreas, ovary, and testis.

3.3.1 Pancreas

The mouse pancreas exhibited a dynamic pattern of expression (Figure 29). A very low level of MI-ER1 cytoplasmic expression was detected in some of the exocrine cells with both the alpha and pan antibodies, indicating that the alpha form is expressed there. However, the expression of MI-ER1 in the endocrine cells of the Islets of Langerhans was extremely intense. Expression was detected with both antibodies and was cytoplasmic in both cases, indicating that the alpha isoform is expressed in the endocrine cells of the Islets.

3.3.2 Thyroid

In the thyroid gland (Figure 30), the colloid, in the centre of the follicle was stained with both antibodies, however this is extracellular staining and is most likely an artifact. Intense cytoplasmic staining was detected with the pan antibody in all the thyroxine and triiodothyronine secreting follicular cells bordering the colloid; anti-pan MI-ER1 detected no nuclear expression, indicating MI-ER1 beta cytoplasmic expression. The parafollicular cells, which do not border the colloid, have larger nuclei and secrete

calcitonin, were stained intensely with the two antibodies. Expression was detected in both the nuclei (25%) and cytoplasm with the pan-antibody and only in the cytoplasm with the alpha antibody. Thus, we can conclude that alpha is expressed in the cytoplasm and beta is expressed in the nuclei (and possibly cytoplasm) of the parafollicular cells.

3.3.3 Adrenal glands

Another endocrine organ, the adrenal gland, expressed MI-ER1 in both its cortex and medulla. In the cells of the zona glomerulosa of the cortex, the proliferative region, intense nuclear and cytoplasmic staining was detected, when using the pan antibody (Figure 31D) while MI-ER1 alpha was exclusive to the cytoplasm (Figure 32D). This shows that MI-ER1 beta is expressed in the cell nuclei, while alpha and possibly beta are expressed in the cell cytoplasm. In the cells of the zona fasciculata, the corticosterone and cortisol secreting zone, both the nuclei (approximately 50%) and cytoplasm were stained with the pan antibody (Figure 31D). However, in the zona fasciculata, there was a small region of cells closest to the outside that was not stained with the pan antibody, thus MI-ER1 was not expressed there. Expression was detected in the cell cytoplasm with the alpha antibody (Figure 32D), indicating that MI-ER1 beta is expressed in the nuclei, while MI-ER1 alpha and possibly beta are expressed in the cytoplasm of cells of the fasciculata. There was a gradient of staining when the alpha antibody was used, with the staining becoming more intense towards the medulla. In the innermost region of the cortex, the x-zone (the zone which secretes sex hormone substrates), the pan antibody detected weak cytoplasmic expression in the cells, while the alpha antibody detected no

expression (Figures 31F and 32F), thus only the beta isoform is expressed in the cells of the x-zone. Finally, in the chromaffin cells of the medulla, there was intense cytoplasmic expression detected with both pan (Figure 31H) and alpha anti-MI-ER1 (Figure 32H), and a medium level of nuclear staining (approximately 50% of the nuclei) was detected with the pan antibody. This indicates that beta is expressed in the nuclei, while alpha is expressed in the cytoplasm of these chromaffin cells.

3.3.4 Testis

In the testis, differentiation of sperm occurs from the outermost region to the lumen of the seminferous tubule. Germ cells progressively differentiate into mature sperm as they move from the outermost layer to the lumen. Thus, one can identify spermatogonia on the periphery of the tubule (close to the tunica propria), spermatocytes in the middle and mature sperm in the lumen of the tubule. In the spermatogonia, approximately 50% of the nuclei and all of the cytoplasm of the cells were stained strongly with the pan antibody (Figure 33D); weak cytoplasmic expression was detected in all the cells with the alpha antibody (Figure 33H). Thus, MI-ER1 alpha and possibly beta are expressed in the cytoplasm and MI-ER1 beta is expressed in the nuclei of the spermatogonia. All the nuclei and the cytoplasm of the spermatocytes were stained at a strong level with the pan antibody; anti-MI-ER1 alpha detected only weak cytoplasmic expression in these cells. This indicates alpha and possibly beta cell cytoplasmic expression and beta nuclear expression. In the spermatozoa, the cell cytoplasm was stained moderately with the pan antibody and weakly with the alpha antibody, demonstrating that MI-ER1 alpha and possibly beta are expressed here.

3.3.5 Ovary

In the ovary, mature ova are generated by oogenesis as the primary follicles develop into secondary follicles and finally into mature Graafian follicles where the oocyte is released. Once this occurs, the follicle degenerates into a corpus luteum. The mouse ovary showed a distinct pattern of expression of both isoforms. In all the granulosa cells of the primary follicle, there was intense nuclear staining detected with the pan antibody (Figure 34D), while expression was not detected with the alpha antibody (Figure 35D), indicating that MI-ER1 beta is only expressed in these cells. The pan antibody detected intense expression in both the cytoplasm and the nucleus in all the granulosa cells from the secondary follicle (Figure 34F), while the alpha antibody detected expression in only the cytoplasm (Figure 35F), indicating that the beta isoform is expressed in the nucleus while the alpha and perhaps beta isoforms are expressed in the cytoplasm of these cells. In the granulosa cells of the secondary follicles, there was a gradient of staining present when the alpha antibody was used, with the staining becoming more intense towards the inner cells closest to the oocyte. Both the antibodies also stained the oocyte (at all stages), indicating that alpha and possibly beta are expressed here. In the ovary, the corpus luteum was also stained. A moderate level of cytoplasmic and nuclear (50% of nuclei) expression were detected in the cells with the pan antibody (Figure 34H), while only weak cytoplasmic expression was found with the alpha antibody (Figure 35H), thus, we can conclude that the beta isoform is expressed in the cell nuclei, while the alpha isoform is expressed in the cell cytoplasm. MI-ER1 beta may or may not be present in the cytoplasm.

Figure 29. Expression of MI-ER1 in the adult mouse pancreas

The pancreas of adult mouse was stained with preimmune sera (A,B) and pan anti-MI-ER1 (C,D) or preimmune sera (E,F) and anti-MI-ER1 alpha (G,H). Boxes shown in A and C correspond to panels B and D while boxes shown in E and G correspond to panels F and H, respectively. Arrows indicate cytoplasmic staining in the islet cells. Imuunostaining appears brown while counterstain appears blue.

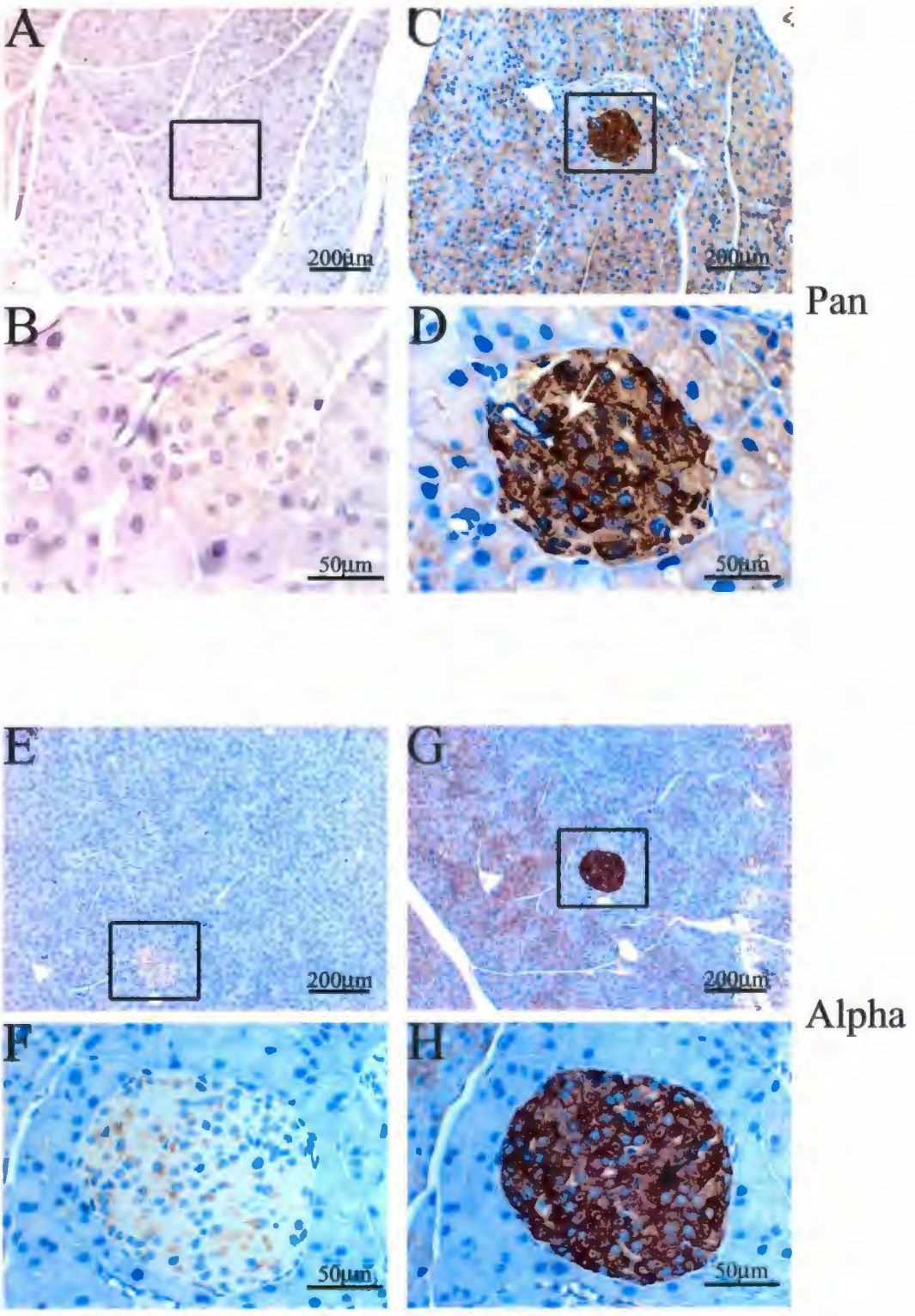

Preimmune
Immune
A
C
200µm
200µm
Pan
B
D
50µm
50µm
E
G
200µm
200µm
Alpha
F
H
50µm
50µm

Figure 30. Expression of MI-ER1 in the adult mouse thyroid.

The thyroid of adult mouse was stained with preimmune sera (A,B) and pan anti-MI-ER1 (C,D) or preimmune sera (E,F) and anti-MI-ER1 alpha (G,H). Boxes shown in A and C correspond to panels B and D while boxes shown in E and G correspond to panels F and H, respectively. Arrows indicate nuclear and cytoplasmic staining in the follicular cells, parafollicular cells, and colloid. Imuunostaining appears brown while counterstain appears blue. C = colloid; FC = follicular cells; PF = parafollicular cells.

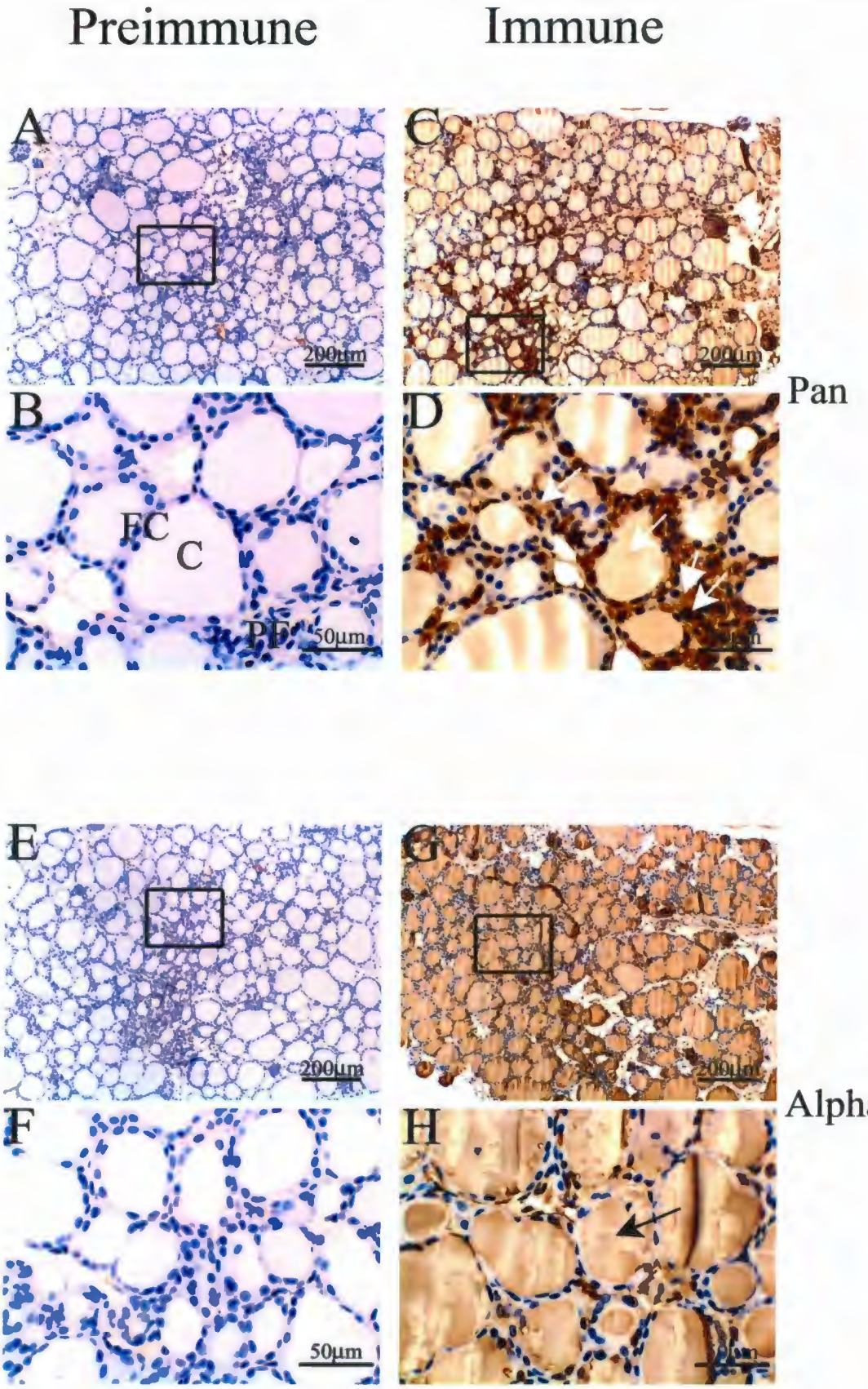

Preimmune
Immune
A
C
200µm
200µm
B
D
FC
C
PF
50µm
Pan
E
G
200µm
200µm
F
H
50µm
Alpha

Figure 31. Expression of MI-ER1 in the adult mouse adrenal gland.

The adrenal glands of adult mouse were stained with preimmune sera (A,C,E,G) and pan anti-MI-ER1 (B,D,F,H). Boxes shown in A correspond to panels C,E, and G while boxes shown in B correspond to panels D,F and H respectively. Arrows indicate nuclear and cytoplasmic staining in cells of the zona glomerulosa and zona fasciculata (D,F) and nuclear and cytoplasmic staining in cells in the medulla (H). Immunostaining appears brown while counterstain appears blue. ZG = Zona glomerulosa; ZF = Zona fasciculata; X = X-zone; M = Medulla.

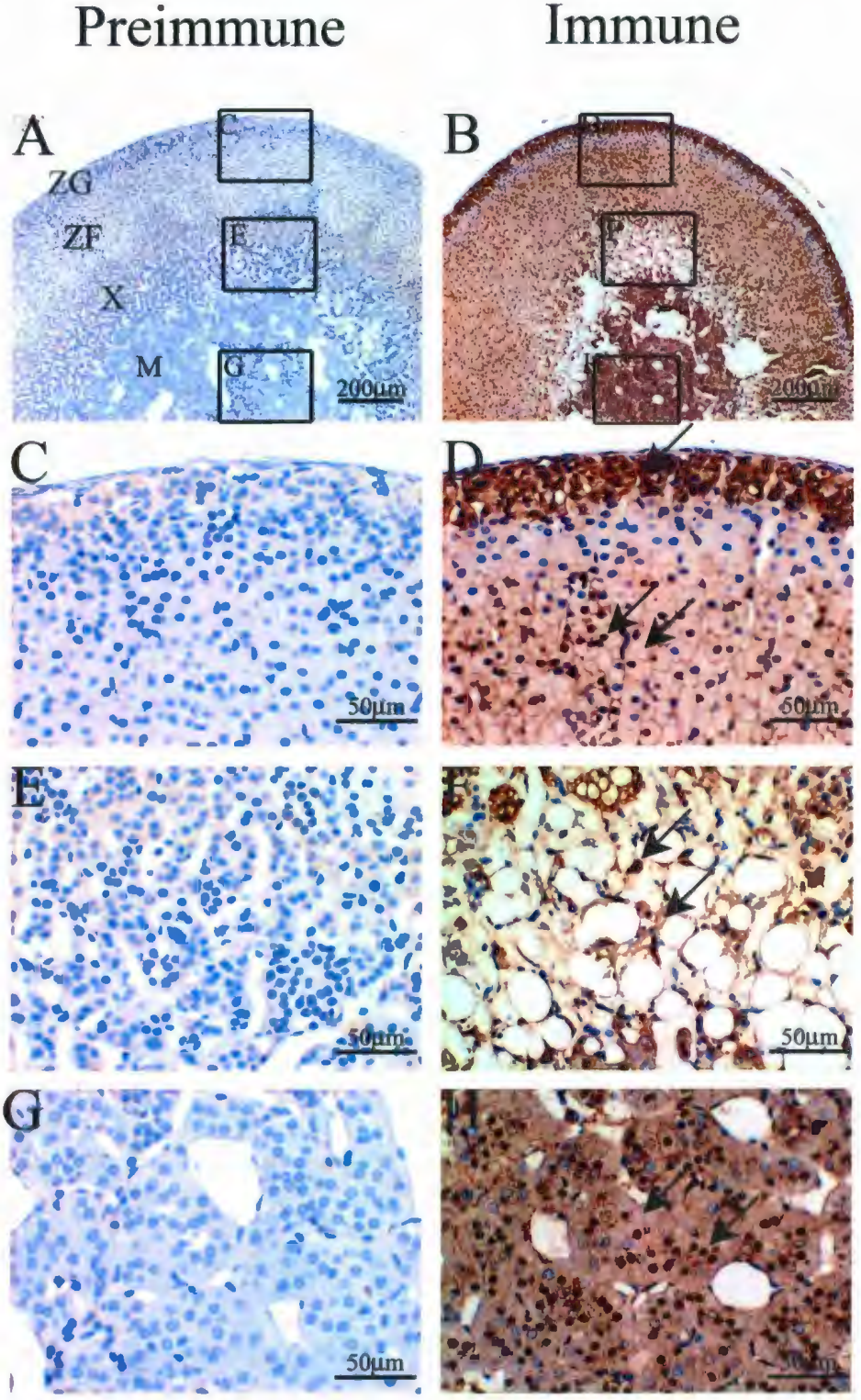

Preimmune
Immune
A
ZG
ZF
X
M
C
E
G
200µm
B
200µm
C
50µm
D
60µm
E
50µm
F
50µm
G
50µm

Figure 32. Expression of MI-ER1 alpha in the adult mouse adrenal gland

The adrenal glands of adult mouse were stained with preimmune sera (A,C,E,G) and anti-MI-ER1 alpha (B,D,F,H). Boxes shown in A correspond to panels C,E, and G while boxes shown in B correspond to panels D,F and H respectively. Arrows indicate cytoplasmic staining in cells of the zona glomerulosa and zona fasciculata (D,F) and cytoplasmic staining in cells of the medulla (H). Immunostaining appears brown while counterstain appears blue. ZG = Zona glomerulosa; ZF = Zona fasciculata; X = X-zone; M = Medulla.

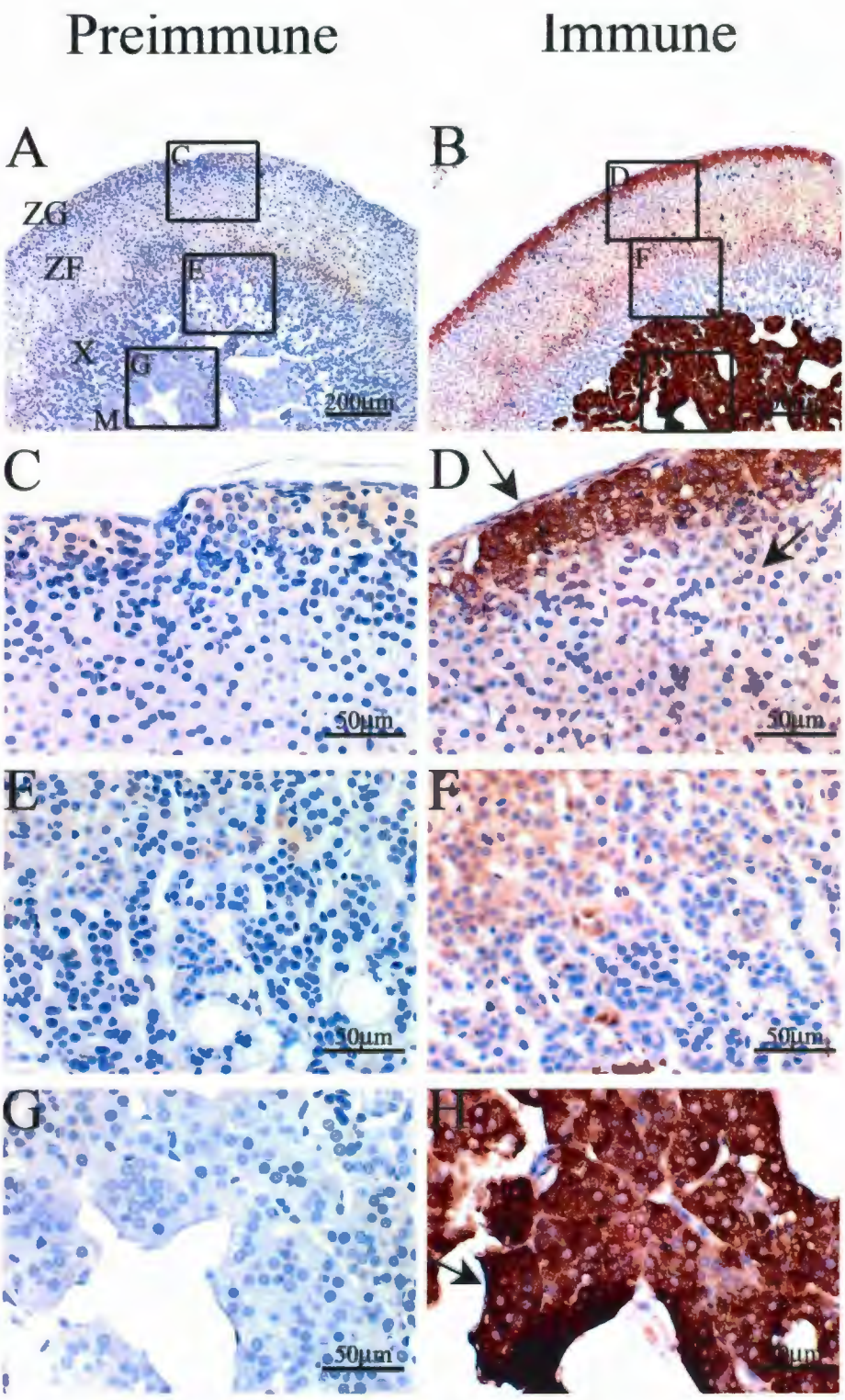

Preimmune
Immune
A
B
C
D
E
F
G
H
ZG
ZF
X
M
200μm
50μm

Figure 33. Expression of MI-ER1 in the adult mouse testis

The testis of adult mouse was stained with preimmune sera (A,B) and pan anti-MI-ER1 (C,D) or preimmune sera (E,F) and anti-MI-ER1 alpha (G,H). Boxes shown in A and C correspond to panels B and D while boxes shown in E and G correspond to panels F and H, respectively. Arrows indicate nuclear and cytoplasmic staining of spermatocytes (D) and cytoplasmic staining of spermatocytes(H). Imuunostaining appears brown while counterstain appears blue.Sg = Spermatogonia; Sc = Spermatocytes; Sz = Spermatozoa.

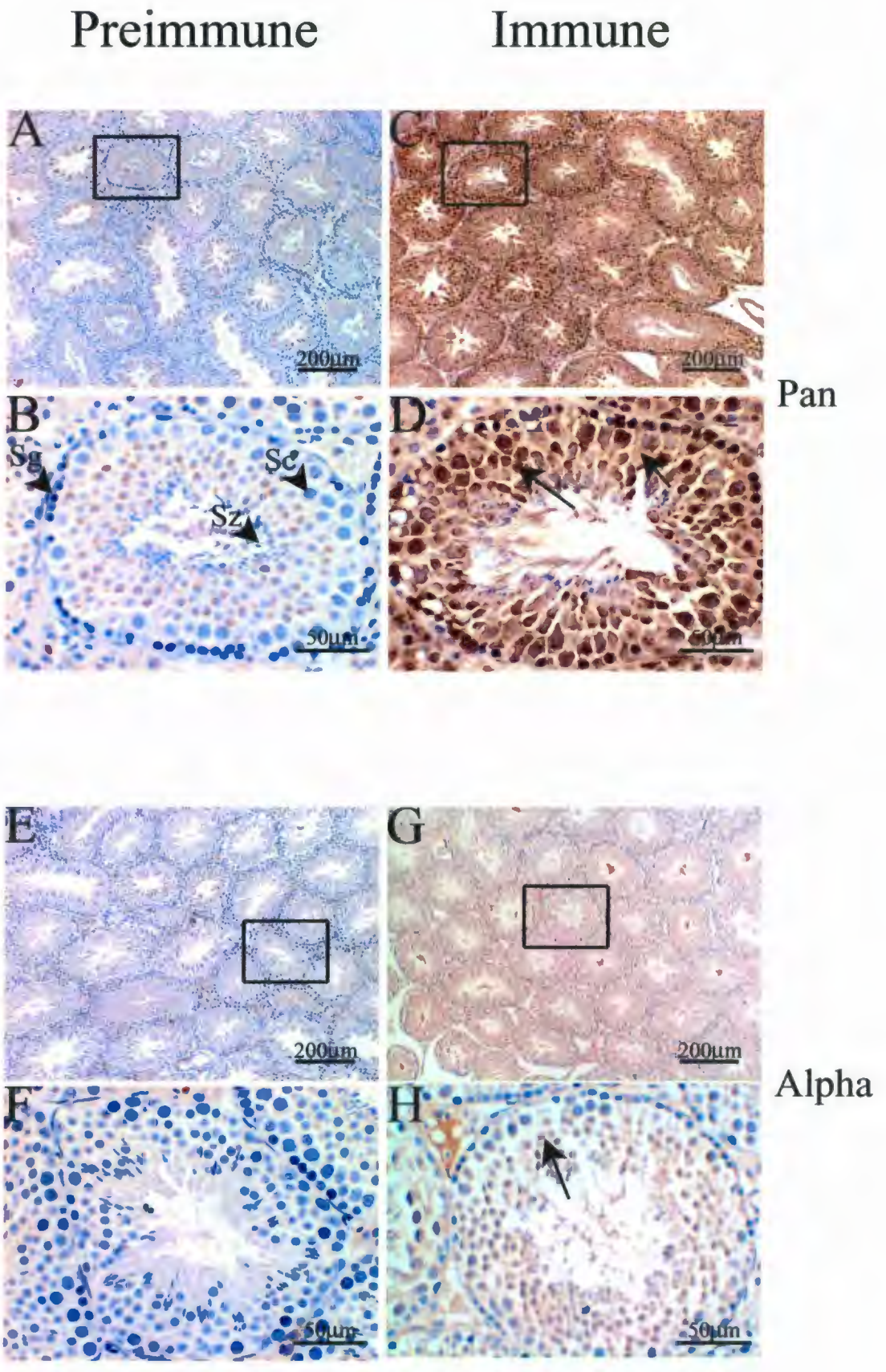

Preimmune
Immune
A
C
200μm
200μm
Pan
B
D
Sg
Sc
Sz
50μm
50μm
E
G
200μm
200μm
Alpha
F
H
50μm
50μm

Figure 34. Expression of MI-ER1 in the adult mouse ovary

Ovary of adult mouse was stained with preimmune sera (A,C,E,G) and pan anti-MI-ER1 (B,D,F,H). Boxes shown in A correspond to panels C,E, and G while boxes shown in B correspond to D,F and H. Primary follicles are shown in C and D; Secondary follicles are shown in E and F; and the corpus luteum is shown on G and H. Arrows indicate staining in the primary follicle (D), cytoplasmic and nuclear staining of the secondary follicle (F) and corpus luteum (H). Immunostaining appears brown while counterstain appears blue. TI= theca interna; GC= granulosa cells; PO= primary oocyte, A=antrum.

Preimmune Immune

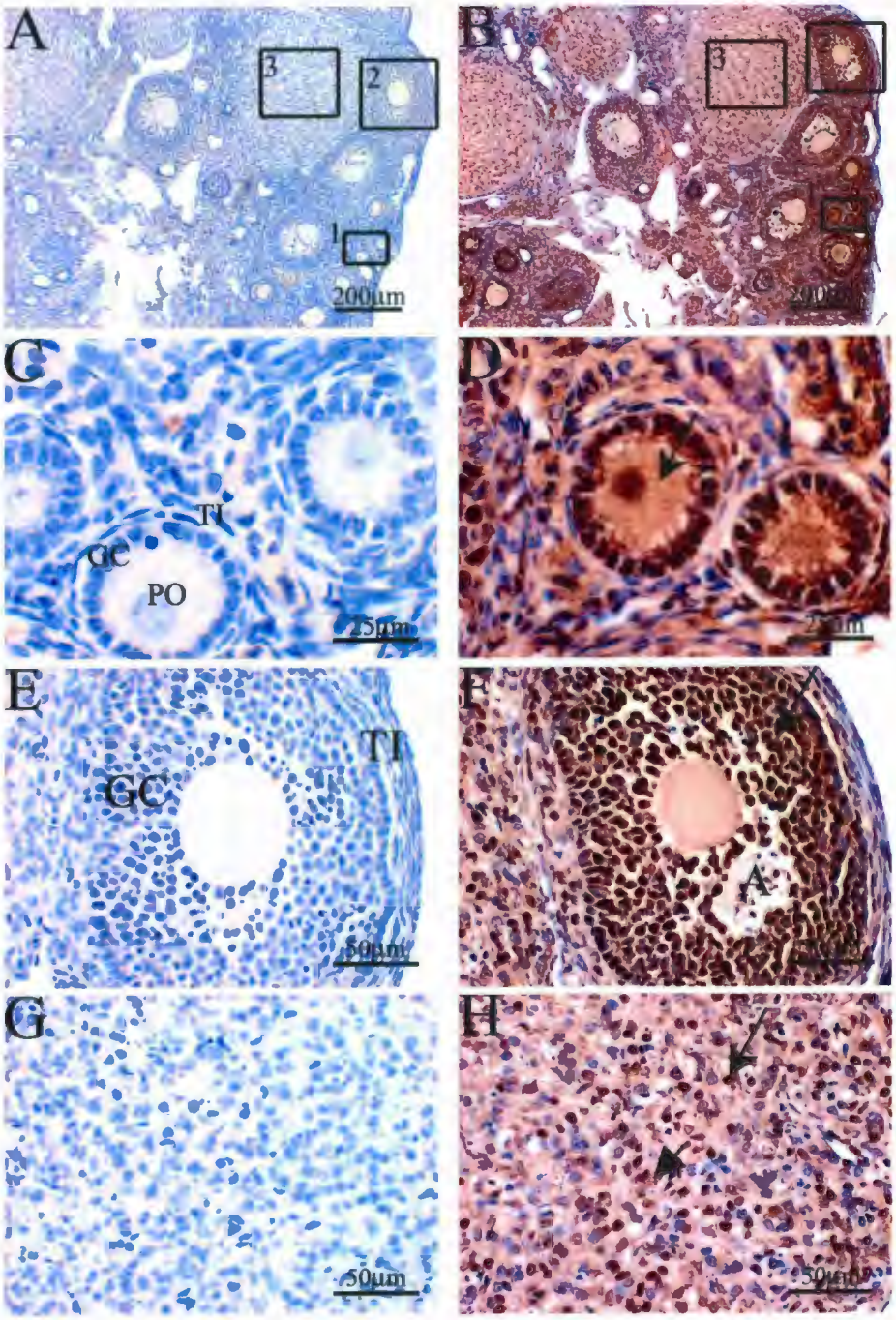
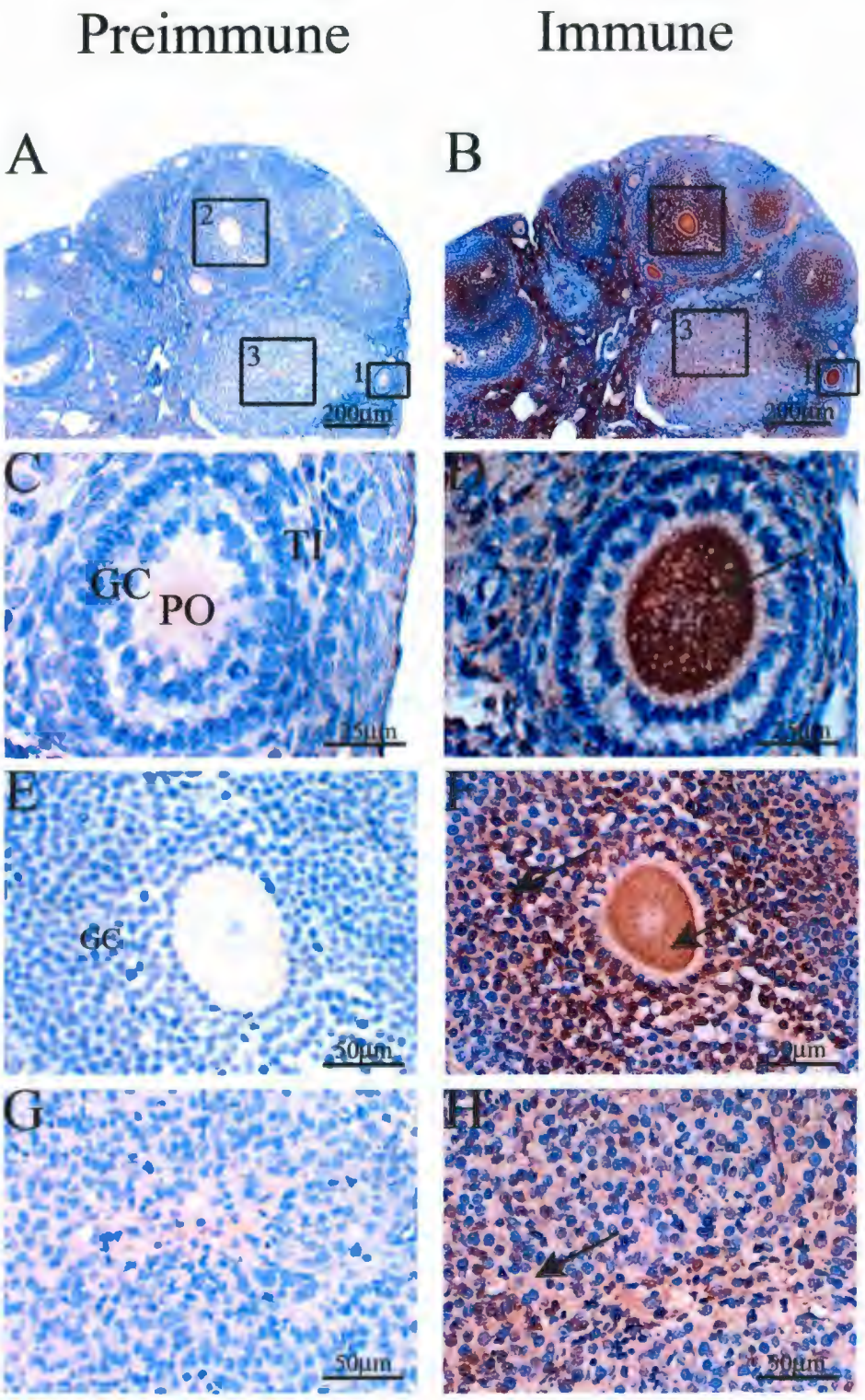


Figure 35. Expression of MI-ER1 alpha in the adult mouse ovary

Ovary of adult mouse was stained with preimmune sera (A,C,E,G) and anti-MI-ER1 alpha (B,D,F,H). Boxes shown in A correspond to panels C,E, and G while boxes shown in B correspond to D,F and H. Primary follicles are shown in C and D; Secondary follicles are shown in E and F; and the corpus luteum is shown on G and H. Arrows indicate staining in the primary follicle (D), cytoplasmic staining of the secondary follicle and oocyte (F) and corpus luteum (H). Immunostaining appears brown while counterstain appears blue. TI= theca interna; GC=granulosa cells; PO= primary oocyte.

.


Preimmune
Immune
A
B
2
3
1
200μm
C
TI
GC
PO
15μm
D
E
GC
50μm
F
50μm
G
50μm
H
50μm

3.4 Results summary

In the 8, 12, and 16-day mouse embryo the alpha isoform was not detected, thus only MI-ER1 beta appears to be expressed in the embryo. Overall, there was decreased expression of MI-ER1 in the adult compared to the embryo. Furthermore, there was a pronounced difference in MI-ER1 subcellular localization between many tissues in the embryo and tissues in the adult (**Tables 1, 2 and 3**). There was both nuclear and cytoplasmic expression of MI-ER1 beta in cells of the embryonic brain and liver, while there was cytoplasmic expression of MI-ER1 alpha (and possibly MI-ER1 beta) in the cells of the corresponding adult tissue. There was a change in the subcellular localization of MI-ER1 from the embryo to the adult in cells of the skeletal muscle, heart, lung and digestive tract. In these embryonic tissues, MI-ER1 beta was expressed in the cytoplasm of the cells, while MI-ER1 alpha was expressed in the cytoplasm of the corresponding adult cells.

The adult mouse spleen was the only adult tissue where there was no detectable expression of MI-ER1 alpha. Furthermore, the spleen was the only adult tissue where MI-ER1 beta was clearly expressed in the cytoplasm. In the other adult tissues that had cell cytoplasmic staining with the pan antibody, there was also staining with the alpha antibody. Thus, the MI-ER1 beta isoform could be expressed in the cytoplasm with the alpha isoform in the cells, but it is also equally possible that only alpha was expressed.

In the cells of the adult tissues examined, there was an overall weak level of MI-ER1expression, with the exception of the endocrine tissues. Interestingly, the endocrine organs showed a particularly high level of MI-ER1 expression. The thyroid, pancreas,

adrenal gland, ovary, and hypothalamus expressed both MI-ER1 beta and alpha. Of note, there was a particularly high level of MI-ER1 alpha expression compared to the other non-endocrine adult tissues that were examined. Thus, MI-ER1 alpha is more highly expressed in endocrine tissues than non-endocrine tissues of adult mice.

Table 1: Location of pan MI-ER1 and MI-ER1 alpha expression in the 16-day mouse embryo

TISSUE	PAN ANTI-MI-ER1 STAINING			ANTI-MI-ER1 ALPHA STAINING		
	Cell nuclei	% cell nuclei	Cell cytoplasm	Cell nuclei	% of cell nuclei	Cell cytoplasm
Brain	+++	75	+++	0	0	0
Skeletal muscle	0	0	+++	0	0	0
Heart	0	0	+++	0	0	0
Lung	0	0	+++	0	0	0
Digestive Tract	0	0	+++	0	0	0
Liver	+++	50	+++	0	0	0

Key:

0 = no staining
+ = weak staining
++ = moderate staining
+++ = intense staining

Table 2: Location of pan MI-ER1 and MI-ER1 alpha expression in adult mouse tissues

TISSUE	PAN ANTI-MI-ER1 STAINING			ANTI-MI-ER1 ALPHA STAINING		
	Cell nuclei	% cell nuclei	Cell cytoplasm	Cell nuclei	% cell nuclei	Cell cytoplasm
Brain						
Cortex	0	0	++ (75% of cells)	0	0	+ (75% of cells)
Hippocampus	0	0	+++	0	0	++
Thalamus	0	0	+ (60 % of cells)	0	0	+++ (60% of cells)
Hypothalamus	0	0	+	0	0	+++
Skeletal Muscle	0	0	+	0	0	+
Heart	0	0	++	0	0	++
Lung						
Epithelium surrounding bronchi	0	0	+	0	0	+
Alveoli	0	0	-	0	0	-
Digestive Tract	0	0	++	0	0	+
Liver	++	100	++	0	0	+
Kidney						
Cortex						
-Tubules	+	100	++	0	0	++
-Glomeruli	0	0	0	0	0	0
Medulla	++	100	++	0	0	++
Spleen						
Red pulp	+	100	++	0	0	0
White pulp	0	0	++	0	0	0
Pancreas						
Islets of Endocrine	0	0	+++	0	0	+++
Exocrine Portion	0	0	+	0	0	+
Thyroid						
Follicular	0	0	+++	0	0	-

cells						
Parafollicular cells	+++	100	+++	0	0	+++
Adrenal gland						
Cortex						
-Glomer.	+++	100	+++	0	0	+++
-Fasciculata	++	50	++	0	0	+
-Reticularis	0	0	+	0	0	+
Medulla	++	50	+++	0	0	+++
Testis						
Spermatogonia	+++	50	+++	0	0	+
Spermatocytes	+++	100	+++	0	0	+
Spermatozoa	0	0	++	0	0	+
Ovary						
Follicular cells of 1^0 follicle	+++	100	+++	0	0	0
Follicular cells of 2^0 follicle	+++	100	+++	0	0	+++
Corpus luteum	++	50	++	0	0	+

Key:

0 = no staining
+ = weak staining
++ = moderate staining
+++ = intense staining

Table 3: Comparison of subcellular localization of MI-ER1 alpha and beta in embryonic and adult mouse tissues

TISSUE	MI-ER1 ALPHA		MI-ER1 BETA	
	Nucleus	Cytoplasm	Nuclear	Cytoplasm
Embryonic brain	-	-	-	+
Adult brain	-	+	-	?
Embryonic skeletal muscle	-	-	-	+
Adult skeletal muscle	-	+	-	?
Embryonic heart	-	-	-	+
Adult heart	-	+	-	?
Embryonic lung	-	-	-	+
Adult lung	-	+	-	?
Embryonic digestive tract	-	-	-	+
Adult digestive tract	-	+	-	?
Embryonic liver	-	-	+	+
Adult liver	-	+	+	?
Kidney	-	+	+	?
Spleen	-	-	+	+
Pancreas	-	+	-	?
Thyroid	-	+	+	?

Adrenal gland	-	+	+	?
Testis	+	+	+	?
Ovary	-	+	+	?

Key:

+ = Expressed

- = Not expressed

? = Cannot conclude

4. DISCUSSION

MI-ER1expression was detected in the mouse tissues that were analyzed in this study, except the bladder and the eye (data not shown). In these tissues, the preimmune staining was too strong to draw any definite conclusions. It is possible that these tissues would need to be stained with a different antibody concentration as only one concentration could be tested in this study.

4.1 MI-ER1 alpha is not expressed in the mouse embryo

In the mouse embryo, there was no expression of MI-ER1 alpha detected in the 8, 12 or 16-day embryo, while the pan-antibody detected MI-ER1 expression meaning that the only isoform present in the 8, 12 and 16-day embryo is MI-ER1 beta. It is possible, however, that MI-ER1 alpha is expressed at an extremely low level that was below the level of detection. In this case, the expression would be so low that the isoform would likely not play a vital role in the embryo. Also, when MI-ER1 alpha expression could not be detected with the alpha and pan specific antibodies, different antibody concentrations were used and immunostaining experiments were repeated. This ensured that the absence of MI-ER1 alpha detection was not due to the use of an inappropriate antibody dilution.

The observation that the alpha isoform is not expressed in the embryo from 8 to 16 days is extremely important to any future transgenic studies of *mi-er1*. These results show that it may be possible to knock out the alpha isoform without producing an embryonic lethal. The results also tell us that it would not be advantageous to eliminate the beta isoform. Since the isoform was so strongly expressed in the embryo and has

previously been shown to play an important role in *Xenopus* development (Teplitsky *et al.*, 2003), then it is likely that it is playing a crucial role in the mouse embryo as well. Thus, knocking out MI-ER1 beta would likely produce an embryonic lethal.

Other studies involving knockouts of the FGF signaling pathway, a pathway that involves MI-ER1, have given us important insights into mammalian embryonic development. Studies using FGF ligand and receptor knockouts have shown that this signaling pathway is important in gastric (Spencer-Dene *et al.*, 2006), kidney (Poladia *et al.*, 2006), and limb development (Li *et al.*, 2005); mesenchymal and epithelial proliferation (del Moral *et al.*, 2006); as well as other stages in mammalian development. Given the results of these studies, future development of MI-ER1 knockout mice may play an important role in further elucidating the mechanisms of embryonic development involving the FGF signaling pathway.

4.2 Subcellular localization of MI-ER1 beta in the mouse embryo

In the embryonic tissues, MI-ER1 beta was found to be expressed in the cytoplasm of the various cell types examined. This was a surprising result since MI-ER1 beta contains the only functional NLS and is usually found in the nucleus. However, in early stages of *Xenopus* development, MI-ER1 beta is retained in the cytoplasm through binding to a cytoplasmic anchor protein (Luchman *et al.*, 1999). Therefore, it is possible that MI-ER1 beta is retained in the cytoplasm in the mouse embryo to regulate its function, keeping it from the nucleus so that it cannot affect the transcription of other genes. It is also possible that MI-ER1 beta is found in the cytoplasm of adult tissues.

4.3 Subcellular localization of MI-ER1 beta in adult mouse tissue

In the adult, MI-ER1 beta was expressed in cell nuclei and MI-ER1 alpha was expressed in cell cytoplasm with the exception of the testes and spleen. In the spleen, like the embryo, MI-ER1 beta was expressed in both the nuclei and the cytoplasm of the cell types examined. Therefore, it is possible that MI-ER1 beta is anchored in the cytoplasm of the red and white pulp cells of the spleen to regulate the amount of MI-ER1 beta available to enter the nucleus. Furthermore, it is possible that the beta isoform has left the nuclei of cells in the spleen to enter a degradation pathway in the cytoplasm. The transcription factor FOXO is regulated in this way. In the presence of growth factors, FOXO is phosphorylated by Akt (Biggs *et al.*, 1999; Brunet *et al.*, 1999; Kops and Burgering, 1999; Nakae *et al.*, 1999; Rena *et al.*, 1999; Tang *et al.*, 1999), which results in the translocation of FOXO to the cytoplasm (Biggs *et al.*, 1999; Brunet *et al.*, 1999; Takaishi *et al.*, 1999) where it is degraded by the ubiqutin proteasome pathway (Matsuzaki *et al.*, 2003; Plas and Thompson, 2003; Aoki *et al.*, 2004; Hu *et al.*, 2004; Huang *et al.*, 2005). Since MI-ER1 beta and FOXO are transcription factors that are involved in processes such as the cell cycle and cell proliferation, it possible that the MI-ER1 beta protein is regulated in a similar manner as FOXO.

4.4 MI-ER1 is not expressed in the nuclei of cells in certain adult tissues

There were six adult mouse tissues where neither MI-ER1 alpha or beta were expressed in the cell nuclei: the adult brain, heart, lung, skeletal muscle, intestine and pancreas. This is surprising since MI-ER1 is a transcription factor that needs to be in the

nucleus to affect the transcription rate of other genes. Cytoplasmic sequestering has been found in other transcription factors as well. For example, unphosphorylated Smad3 is sequestered in the cytoplasm through the binding of Akt, which prevents Smad3 mediated transcription (Conery *et al.*, 2004). It is therefore possible that MI-ER1 is sequestered in the cytoplasm to inhibit its function.

4.5 MI-ER1 expression in the embryonic and adult mouse brain

In the embryonic mouse brain only MI-ER1 beta was expressed and this expression was both cell nuclei and cytoplasm. However, in adult mouse MI-ER1 alpha became expressed in the cell cytoplasm (possibly with MI-ER1 beta), and MI-ER1 beta was no longer expressed in the nuclei. This suggests the MI-ER1 alpha isoform becomes expressed during the development of the mouse and this must occur after embryonic day 16. The mechanism of how and why MI-ER1 alpha becomes expressed in the adult, along with determining the specific adult subcellular localization of the MI-ER1 beta protein in the adult mouse brain would be studies to conduct in the future.

There was a striking pattern of MI-ER1 expression in the adult brain. MI-ER1 expression was found in cells of the hippocampus, a region of the brain that stores episodic memories (memories that can be formed during a single occurrence, can be articulated and are linked to a particular context) (Lisman, 1999). Particularly, MI-ER1 expression was exclusive to the CA3 cells, a heteroassociative network that links different memories that occurred at different times. Other transcription factors have also been found to play an essential role in this area of the brain. For example, the cyclic

AMP response element-binding protein (CREB) protein is expressed in the hippocampus and has been implicated in long-term memory (Brightwell *et al.*, 2007). Given the fact that the CREB protein binds to CBP (Gonzalez and Montminy, 1989) and that CBP binds to MI-ER1 (Blackmore, 2004), these three proteins may form a complex in the CA3 cells of the hippocampus and function in the formation of long-term memories.

The brain is an important target for steroid hormones, therefore it expresses nuclear steroid receptors including the estrogen receptor, progesterone receptor, androgen receptor, glucocorticoid receptor and mineralocorticoid receptor. Their expression is particularly high in the hippocampus and hypothalamus (Kawata *et al.*, 1998). Given that MI-ER1 interacts with nuclear steroid receptors, it is possible that it is interacting with these receptors in these areas of the brain.

4.6 MI-ER1 expression in the embryonic and adult mouse liver

There was an intriguing result regarding the MI-ER1 expression in the embryonic liver. In this tissue, two distinct cell types were identified. One cell type expressed MI-ER1, while the other cell type was negative for MI-ER1 expression. It is possible that the cell that does not express MI-ER1 is a hematopoetic cell while the other cell is a hepatocyte. This hypothesis is based on the morphology of the cell types and the fact that the liver is the main hematopoietic organ from embryonic day 10 to 15 in the mouse embryo (Sasaki and Sonoda, 2000). However, confirmation of this theory would require immunostaining the embryonic tissue for markers present in hematopoetic cells and markers present in hepatocytes.

4.7 MI-ER1 expression in the adult mouse kidney and intestine

The MI-ER1 protein was present in the adult mouse kidney and intestine, with nuclear and cytoplasmic in the cells of the kidney and exclusively cytoplasmic expression in the intestinal cells. One possibility why MI-ER1 would be expressed in these tissues is that it may be interacting with the vitamin D receptor (VDR), a member of the superfamily of steroid/thyroid hormones. The VDR is responsible for converting vitamin D to its active form of 1,2,5 dihydroxyvitamin D where it exerts its affects on target organs. Since the VDR is a nuclear steroid hormone receptor, it possible that it is interacting with this receptor in the cells of the kidney and intestine. In addition, the VDR is not expressed in the podocytes of the glomeruli (Liu *et al.*, 2006), which were the same cells that did not express MI-ER1. This further supports the theory that MI-ER1 is interacting with the VDR in the kidney since MI-ER1 is expressed in the epithelial cells of the tubules, the same cells that express VDR (Liu *et al.*, 2006).

4.8 MI-ER1 expression in the adult mouse spleen

The spleen is the major site of immune response to blood-borne antigens and it is an important filter for blood, whereby the red pulp macrophages clear the blood of microbes (Abbas, 2000). In this study, expression of MI-ER1 beta was found in the nuclei and cytoplasm of the cells of the red and white pulp. No expression was detected with the alpha antibody. This was a striking result because the spleen was the only adult tissue where we could definitely conclude that MI-ER1 beta was expressed in the cell

cytoplasm. This may be because it binds to a cytoplasmic anchor, which prevents it from entering the nucleus. As mentioned previously, it may also translocate to the cytoplasm where it is degraded, thereby regulating its function. The reason this occurs in the spleen would be interesting to investigate and may provide us with other functions of the MI-ER1 protein. Furthermore, the spleen was the only adult tissue that did not express the MI-ER1 alpha protein. Finally, in this study it was not possible to distinguish between the T and B lymphocytes since they do not differ morphologically. Thus, to determine the exact types of cells that are expressing MI-ER1, specific markers of T and B lymphocytes and macrophages need to be immunostained to determine the difference in the cell types.

4.9 MI-ER1 expression in adult mouse endocrine and endocrine responsive tissues

The expression of MI-ER1 was detected in the endocrine organs as well. In these organs, the expression of MI-ER1 alpha was much more intense compared to other adult tissues. Thus, MI-ER1 alpha may play a vital role in these organs. This is not surprising given that all these organs express nuclear steroid receptor proteins.

4.9.1 Pancreas

The expression of MI-ER1 in the pancreas was quite striking. In this tissue, intense cytoplasmic expression of MI-ER1 alpha (possibly beta) was detected in the cells comprising the Islets of Langerhans, the endocrine portion consisting of alpha, beta and delta cells which secrete glucagons, insulin and somatostatin, respectively. Given the association of MI-ER1 alpha with nuclear steroid receptors (Savicky, 2004), and the

cytoplasmic expression, it is very likely that the alpha isoform is mainly expressed in the endocrine cells of Islets. It is very probable that MI-ER1 alpha is binding to nuclear steroid receptors in these endocrine cells and regulating their function. There was MI-ER1 expression detected in the exocrine portion of the pancreas, the portion that is responsible for secreting digestive juices into the small intestine (Carola, 1990). This expression was only cytoplasmic and also was not uniform. The reason for this and the function of MI-ER1 in the pancreas remains to be investigated.

The differences in the exocrine and endocrine portions of the pancreas were identified by morphology in this study. However, a more specific method could be used to definitely distinguish these regions. To do this, one could stain for a specific marker in the Islets of Langerhans, such as the pancreatic islet-specific glucose-6-phosphatase-related protein (Petrolonis *et al.*, 2004), which catalyzes the dephosphorylation of glucose-6-phosphate to glucose. Thus, use of a specific marker would aid in a more specific and accurate examination of MI-ER1 in the pancreas.

4.9.2 Thyroid

The parafollicular cells of the thyroid expressed the MI-ER1 protein; expression of MI-ER1 beta was detected in the nuclei (possibly cytoplasm), while MI-ER1 alpha was expressed in the cytoplasm. These cells are responsible for secreting the hormone calcitonin, which lowers the calcium level in the blood. The role of MI-ER1 in the thyroid gland has yet to be established, however, the function of other transcription factors in this gland has been examined. For example, the peroxisome proliferators

activator receptor (PPAR) alpha is a transcription factor that is related to steroid, retinoid and thyroid nuclear receptors (Cuzzocrea *et al.*, 2006). This transcription factor recruits other cofactors and coactivators, such as the CREB binding protein (CBP), to form the TRAP (thyroid hormone receptor-associated protein) mediator complex, which facilitates interaction with RNA polymerase II and the basal transcriptional machinery (Issemann and Green, 1990; Dreyer *et al.*, 1992; Rachez *et al.*, 1999; Ranish *et al.*, 1999; Reddy and Hashimoto, 2001; McKenna and O'Malley, 2002; Lewis and Reinberg, 2003; Roeder, 2003; Wang *et al.*, 2003; Miyano, 2005; Cuzzocrea *et al.*, 2006). Thus, since MI-ER1 interacts with the estrogen receptor, a nuclear steroid hormone receptor (Savicky, 2004), CBP (Blackmore, 2004) and has been found in multiprotein complexes, it is also possible that it interacts in the TRAP mediator complex in the thyroid gland. This would be an interesting investigation to undertake in the future.

4.9.3 Adrenal glands

There was also an interesting pattern of expression in the adrenal glands. The adrenal gland is portioned into two distinct regions: the cortex and the medulla. The medulla is fairly homogeneous, however, three distinct zones can be identified in the cortex and each zone produces a different type of hormone. The zona glomerulosa is the thin outermost zone; it supplies cells for all three zones of the cortex. Also, it contains the enzymes necessary for the production of the mineralcorticoid, aldosterone, a steroid hormone synthesized from cholesterol that helps maintain the body's electrolyte balance (Carola, 1990). Beneath the glomerulosa is the zona fasciculata, the layer that makes up

the bulk of the cortex layer and secretes the hormones corticosterone and cortisol as well as a small amount of gonadocorticoids. This layer also has the greatest concentration of cholesterol and considerable amounts of vitamin C. The deepest layer of the cortex is the x-zone, which secretes dehydroepiandrosterone (DHEA), a substrate for the production of the sex hormones. Finally the medulla, which is located in the center of the adrenal gland, secretes epinephrine and norepinephrine, the "fight or flight" hormones.

In the mouse medulla there was an extremely high level of the MI-ER1 alpha protein present in the cell cytoplasm. In the cortex, the cells of the zona glomerulosa expressed MI-ER1, mainly in the cytoplasm, but also in the nuclei, suggesting that both isoforms are present; the MI-ER1 beta isoform is expressed in the nucleus (and perhaps in the cytoplasm) and the MI-ER1 alpha isoform is expressed in the cytoplasm. There was a gradient of staining present in the zona fasciculata (the second region of the cortex), with the greatest level of intensity towards the center. Again, in this region the MI-ER1 beta isoform was expressed in the cell nuclei and the alpha isoform was expressed in the cell cytoplasm. The staining pattern in the adrenal gland was not expected. It was expected that the greatest level of staining would be in the cortical region since it produces steroid hormones and the MI-ER1 alpha protein interacts with nuclear steroid receptors (Savicky, 2004). However, recent evidence suggests that there may be more of a connection and interaction between the cortex and the medulla than previously believed and that they are no longer considered independent endocrine systems (Schinner and Bornstein, 2005). For example, catelcholamines that are released from the cortex increase the transcription rate of steroid enzymes thereby increasing the

synthesis and secretion of the corticosteroids (Ehrhart-Bornstein *et al.*, 1991; Guse-Behling *et al.*, 1992). Thus, it would be possible that the steroid receptor is actually located in the medulla, interacting with the MI-ER1 alpha protein, therefore, it would make sense that the expression of MI-ER1 is higher in this region compared to the cortex. This hypothesis would require further investigation.

4.9.4 Ovary

MI-ER1 expression was detected in the ovary, an organ that is both responsive to and secretes the hormone estrogen and progesterone. The ovary expresses both the estrogen receptor alpha and beta (Tremblay *et al.*, 1997) and these receptors play vital roles in follicle development. In fact, it was found that the expression of estrogen receptor beta is critical for the differentiation of the granulosa cells and the response of these cells to the gonadotropin hormone (Couse *et al.*, 2005). Given the important role for estrogen receptor beta in the granulosa cells, that MI-ER1 was expressed there and that MI-ER1 interacts with the estrogen receptor beta, it is possible that MI-ER1 may play a role in the process of folliculogenesis in the ovary. Also, the transcription factor CEBP beta has been implicated in follicular development as well (Sterneck *et al.*, 1997) . CEBP beta is required for granulosa cell differentiation in response to the lutenizing hormone (LH) and is an important downstream target of LH receptor signaling. Thus, the fact that a transcription factor is involved in folliculogenesis, coupled with the other evidence mentioned above makes it possible that MI-ER1 is involved in this process somehow.

Furthermore, in the secondary follicles, there was a gradient of staining present in the granulosa cells, with staining becoming more intense towards the oocyte. This gradient of staining may occur due to chemical secretion from the oocyte. During development and growth of the follicle, there are many critical cellular interactions that occur. For example, interactions with the ligand KitL (expressed in the granulosa cells) and growth differentiation factor-9 (GDP9) and bone morphogenetic protein-15 (BMP-15) (expressed in the oocyte) activate the TGF-beta signaling pathway. This signaling cascade leads to the activation of Smad proteins, which translocate to the nucleus and interact with transcription factors that regulate gene expression (Lagna *et al.*, 1996; Liu *et al.*, 1997; Nishimura *et al.*, 1998). Overall, this pathway promotes the development of the follicle by promoting the proliferation of the granulosa cells (Hayashi *et al.*, 1999; Otsuka *et al.*, 2000; Vitt *et al.*, 2000), inhibiting progesterone and increasing estradiol production (Vanderhyden and Macdonald, 1998), and inhibiting lutenization (Shimasaki *et al.*, 2004). These factors that are secreted from the oocyte and granulosa cells and initiate this signaling pathway may also cause the gradient in MI-ER1 expression. If this is the case, MI-ER1 may be involved in the process of follicle development in the ovary.

4.9.5 Testis

In the mouse testes, there was a high level of MI-ER1 expression that was detected with the MI-ER1 pan antibody. This result was expected since the testis is an endocrine tissue that has been shown previously to express MI-ER1 alpha (Grant, 2004). The novel isoform, N4 beta, was also found in the testis and was exclusive to this organ

(Thorne *et al.* 2005). Furthermore, it is very possible that MI-ER1 is interacting with the androgen receptor in the testis, since it interacts with nuclear steroid hormone receptors. The androgen receptor mediates many different physiological and development processes in the male, including sexual differentiation and maintaining spermatogenesis. The androgen receptor is expressed in the spermatogonia (Kimura *et al.*, 1993; Zhou *et al.*, 1996), spermatocytes (Kimura *et al.*, 1993) and the elongated spermatids (Vornberger *et al.*, 1994), which was where MI-ER1 expression was found. Thus, it is very possible that MI-ER1 is interacting with this receptor in the testes and regulating the process of spermatogenesis.

4.10 Limitations of Immunohistochemical Analysis

Although immunohistochemical analysis is a powerful tool that can provide information on the temporal and spatial expression of proteins, there are certain limitations to this type of study. For example, often tissues are not stained uniformly due to incomplete antigen retrieval. In this study, the antigen retrieval was performed according to instructions given by the company that prepared the tissue slides and it was found that this method was extremely effective. However, there were times when the tissue was not stained uniformly, with the center staining more intensely that the outside of the sections. Thus, it is possible that this would affect the analysis of the percentage of cell nuclei stained. Furthermore, immunohistochemisty is a qualitative method of determining protein expression and thus, one cannot use this analysis to quantify how much protein is present. In order to do this, a quantitative immunoassay would need to

be employed. Despite having disadvantages, immunohistochemisty is extremely useful in providing one with information on where and when a protein is functioning and can give important information that can lay the groundwork for potential discoveries. For these reasons immunohistochemical analysis will continued to be used in the future for scientific research.

5. CONCLUSIONS

1. The MI-ER1 protein was expressed in all the tissues that were analyzed. It is expressed at particularly high levels in the endocrine tissues (adrenal gland, pancreas, ovary, testes, thyroid and hypothalamus) where it is likely interacting with nuclear steroid receptors. In particular, MI-ER1 alpha was detected at a much higher level in the endocrine tissues compared to other adult tissues.

2. MI-ER1 alpha expression was not detected in the 8, 12 or 16-day embryo, therefore it may be possible to knockout this isoform when constructing transgenic mice. This work would be very significant to the study of MI-ER1 and potentially in the treatment of breast cancer.

3. MI-ER1 beta is therefore the only isoform present in the embryo, thus it is very likely that it plays a crucial role in the development of the mouse. Therefore, it is probable that it could not be knocked out without generating an embryonic lethal and a conditional knockout of this isoform would be necessary to determine any potential functions.

4. Overall, there was a significant decrease in MI-ER1 expression in the adult tissue compared to the embryo.

5. In the embryo, MI-ER1 beta is expressed in the cell nuclei and cell cytoplasm of different cell types in a variety of developing tissues, while MI-ER1 beta is expressed in the cell nuclei and MI-ER1 alpha is expressed in the cell cytoplasm in cells of mouse adult tissues (brain, liver, kidney, thyroid, adrenal, testis and ovary). One exception was

the spleen, which was the only organ we could definitely conclude that MI-ER1 beta was expressed in cell nuclei and cytoplasm.

6. MI-ER1 was not expressed in the nuclei of cells of five mouse tissues that were analyzed. These included adult skeletal muscle, heart, lung, intestine and pancreas. This could possibly be a mechanism to regulate the function of MI-ER1.

In conclusion, this project has been an important in determining the spatial and temporal MI-ER1 expression in the embryonic and adult mouse and it has given us irnportant information on possible functions of this protein. Furthermore, this was a critical preliminary study to the construction of MI-ER1 transgenic mice. These studies will enable us to further investigate the many functions of this protein.

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