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14. ABSTRACT

Yeast two-hybrid system was used to identify MMP interacting proteins as potential novel substrates. The hemopexin domain of MMP-3 interacted with the C-terminus of Wnt5b in a two-hybrid assay. A deletional analysis of this clone revealed a minimal MMP-3 binding domain of 55 amino acids. A hinge/blade swapping approach with MMP-10 further revealed that the hinge region and 3 of the 4 MMP-3 hemopexin domain blades are required for binding of Wnt5b. The C-terminus of Wnt5a is 84% identical to Wnt5b and is the only other Wnt expressed in the mammary gland that interacts with MMP-3 in the two-hybrid assay. Recombinant MMP-3 catalytic domain can cleave both soluble Wnt5b as well as cell bound Wnt5a and b immunoprecipated from RIPA cell lysates. Cleavage seems to occur within the minimal MMP-3 interacting domain as judged by mobility shift on SDS-PAGE and can be blocked by the MMP inhibitors GM-6001 and EDTA. These observations suggest that both Wnt5a and b are MMP-3 substrates, but only Wnt5b is co-expressed with MMP-3 in the ductal microenvironment during mammary gland development. A novel lentiviral based method is being developed to analyze the role of Wnt5a and b and their MMP-3 cleavage products during mammary gland development and/or carcinogenesis.

15. SUBJECT TERMS
Breast cancer formation and progression, matrix metalloproteinases, Substrate identification, yeast two-hybrid assay

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Introduction

Matrix metalloproteinases (MMPs) are a family of extracellular zinc-dependent endopeptidases that have been implicated in a wide range of both physiological and pathological processes. Their involvement in cancer development was initially thought to be restricted to tumor progression, due to their pro-angiogenic activity and potent ability to degrade extracellular matrix (ECM) barriers that normally prevent invasion and metastasis. However, targeted expression of autoactivated rat Stromelysin-1/MMP-3 in mouse mammary glands was shown to induce hyperplasias and breast cancer, demonstrating that this MMP can influence tumor initiation and alter neoplastic risk (Sternlicht *et al.*, 1999). Some insight into the molecular mechanism by which this protease can modify cell behavior came from a recent study by Radisky *et al.* (2005), who identified Rac1b as an intracellular mediator of MMP-3 induced epithelial-mesenchymal transition (EMT) and genomic instability in cultured SCp2 mouse mammary epithelial cells. The key *in vivo* substrates for this MMP remain unknown and it is still unclear how this protease can promote breast cancer. This research project aimed to identify MMP-3 interacting proteins as potential novel substrates. Here I report my findings for the third year of this postdoctoral traineeship award and briefly summarize my findings of the previous two years.

Body

Task 1a, b, c, d and e: Identification of Stromelysin-1/MMP-3 interacting proteins from both mouse and human breast tissue, completed (months 1-9). Two-hybrid screens with the catalytic domain, hemopexin domain and full-length MMP-3 from both human and mouse were performed. The results of these screens are summarized in Table 1. It became clear during this study that only the hemopexin domain should be used as 'bait', since the catalytic domain is 'sticky' and the full-length enzymes are poorly folded in the yeast cytoplasm. To further increase the success rate of this approach, one should construct the two-hybrid library from rough-ER bound mRNA to enrich for clones encoding membrane and secreted proteins. Although the vast majority of interacting clones encoded intracellular proteins, two extracellular interacting proteins were identified. One clone contained the last 280 amino acids of ADAMTS-18, but was not further pursued as a potential MMP-3 substrate since its normal physiological role and its potential for dysregulation during malignancy remain unclear. Another clone contained the last 147 amino acids of Wnt5b. This candidate was pursued as a potential MMP-3 substrate, as inappropriate Wnt signaling has been implicated in a variety of cancers including breast cancer (Reya and Clevers, 2005). The yeast two-hybrid interactions between the different MMP-3 and Wnt5b domains are listed in Table 2. Two different subcloning approaches were used to identify the domains responsible for the MMP-3/Wnt5b interaction. A deletional approach of the original two-hybrid clone is shown in Figure 1 and revealed a minimal MMP-3 binding domain of 55 amino acids, which is

flanked by cysteine residues and is one of the least conserved domains among 19 mouse Wnts. A hinge/blade swapping approach with the 67% identical hemopexin domain of Stromelysin-2/MMP-10 is shown in Figure 2 and revealed that the hinge region and 3 of the 4 MMP-3 hemopexin domain blades are required for binding of the Wnt5b C-terminus. The C-terminus of Wnt5a is 84% identical to Wnt5b and is the only other Wnt expressed in the mammary gland that interacts with MMP-3 (Table 3). The C-termini of Wnt2, 4, 6, 7b and 10b did also not interact with the hemopexin domains of MMP-2, -10 and –14. Collectively, these two-hybrid observations suggest that the interaction between MMP-3 and Wnt5a and b is quite specific. Task 1f and two-hybrid screening with MMP-14 were abandoned for reasons outlined in the second progress report.

Task 2: To confirm that Wnt5a and b are MMP-3 substrates (months 9-18).

Task 2a: in progress. A co-expression approach was initially used to try demonstrate cleavage of Wnt5a and b by MMP-3 *in vitro*. To this extent a furin cleavage site was inserted between the pro- and catalytic domain to ensure activation of MMP-3 (Pei and Weiss, 1995), while Wnt5a and b were N-terminally myc tagged and C-terminally HA tagged. The pLNCX2 retroviral backbone from Clontech was modified to enable simultaneous expression of MMP-3 and double tagged Wnt5a or b from the viral 5' LTR. Infection of HEK 293 and NIH 3T3 cells with these viruses was very efficient, but failed to demonstrate cleavage of Wnt5a and b, despite the fact that approximately 50% of all MMP-3 was converted to the 'mature'/active form (data not shown and Figure 3). Reverse zymography and RT-PCR revealed that HEK 293 cells secrete several TIMPs (tissue inhibitors of metalloproteinases) while NIH 3T3 cells secrete high levels of TIMP2, which likely inhibit the 'mature'/active form of MMP-3. Two NIH 3T3 cell lines stably transduced with different small interfering double stranded RNAs (siRNAs) against TIMP2 have been created, but have yet to be used for cleavage and co-immunoprecipitation studies.

Task 2b: in progress. The catalytic domain of MMP-3 and the full length enzyme were expressed in *E.coli* and solubilized from inclusion bodies. Figure 4 shows the purification and refolding of recombinant MMP-3 catalytic domain. I have thus far been unable to obtain full length enzyme, as it sheds the hemopexin domain during the refolding process. Refolding these proteins by dropwise dilution in the presence of the general MMP inhibitor 1,10-phenantroline may prevent auto-degradation. Large-scale expression and purification of recombinant Wnt5a and b has not been attempted for reasons outlined in the second progress report. Concentrated cell culture supernatant and RIPA lysates derived from cell lines stably expressing single and double-tagged Wnt5a and b were used as substrates in stead. Recombinant MMP-3 catalytic domain was found to cleave cell bound Wnt5a and b immuno-precipitated from RIPA cell lysates (Figure 5) as well as soluble Wnt5b (Figure 6). Cleavage seems to occur within the minimal MMP-3 interacting domain as judged by mobility shift on SDS-PAGE and can be blocked by the MMP inhibitors GM-6001 and EDTA. It will be necessary to confirm these observations with full length enzyme, especially since the hemopexin domain is involved in binding of the Wnt proteins. The cleavage products still need to be purified by immunoaffinity chromatography using agarose immobilized anti-myc and anti-HA

antibodies to identify the cleavage sites by electron spray mass spectroscopy. A human ScFv (single chain antibody fragment) phage display library will subsequently be screened with small antigenic peptides specific for either (cleaved) Wnt5a or b in order to obtain antibodies against the full length and processed forms of these proteins. I intend to establish substrate specificity by co-expressing tagged Wnts with MMPs in NIH 3T3 cells knocked down for TIMP2.

Task 2c: in progress. Hosein Kouros-Mehr, a talented MD/PhD student in our lab, dissected out terminal end buds (TEBs) and ducts from mammary glands of five week old virgin mice and compared their transcriptional profile with that from distant stroma to identify transcripts that are specifically upregulated in the different structures of the mammary gland. Figure 7 shows that Wnt5b and MMP-3 expression was restricted to the ductal microenvironment, while the Wnt5a transcript was only present in the TEB microenvironment. These observations were confirmed by *in situ* hybridization and suggest that Wnt5b could be a genuine MMP-3 substrate *in vivo*, as both genes are expressed at the same time and in the same compartment of the mammary gland. Studies to detect processed Wnts in the mammary gland or to co-immunoprecipitate Wnt5a or b with MMP-3 from mammary gland lysates will be initiated as soon as specific antibodies against these proteins have been generated.

Problems encountered: No significant new progress has been made on any of these Task 2 items over the past year, mainly because I have shifted my focus to try develop a new viral based method to aid gene function studies in the mammary gland (see also Task 3).

Task 3: To establish how processed Wnt5a and b contribute to mammary carcinogenesis (months 19-36)

Task 3a: not yet initiated. Unfortunately I have yet to establish an altered *in vitro* cellular response for both the full length and processed forms of Wnt5a and b. I was still a graduate student working on yeast cell wall biosynthesis when I wrote this research proposal. My inexperience in the breast cancer, MMP and Wnt fields together with an underestimation of how time-consuming mice work can be are probably the main reasons why I did not succeed in adhering to the time line of my original proposal. Nevertheless, I do intend to finish this study and would like to do the following *in vitro* experiments that should provide some insight into how processed Wnt5a and b differ from their full length counterparts.

- Wnts have classically been separated into two classes based on their ability to transform C57mg mouse mammary epithelial cells (Wong *et al.*, 1994). The so-called canonical Wnts are able to transform this cell line by activating the Wnt/β-catenin signaling pathway and include Wnt1, 3a and 8. The non-canonical Wnts 4, 5a and 8 signal through the distinct Wnt/Ca^{2+} pathway and fail to transform this cell line. Activation of the Wnt/Ca^{2+} pathway by Wnt5a seems to be antagonistic to the canonical Wnt/β-catenin signaling pathway in C57mg cells, as endogenous Wnt5a expression maintains these cells in a normal growth state while anti-sense Wnt5a mimics Wnt1 induced transformation

(Olsen and Gibo, 1998). Thus the full length and processed forms of Wnt5a and b can be tested for their ability to block Wnt1 induced transformation of C57mg cells. Alternatively, the luciferase reporter TOPFLASH (Ishitani *et al.*, 1999) can also be used to monitor Wnt1 induced Wnt/β-catenin signaling activity in the presence and/or absence of full length and processed Wnt5a and b.

- It is currently unclear how cleavage of Wnt5a and b by MMP-3 might affect the biology of these molecules. Cleavage could affect bio-availability through loss of plasma membrane association and/or through an inability to bind heparan sulphate proteoglycans. Cleavage could also affect their interaction with secreted antagonists of Wnt signaling, such as the sFRP (secreted Frizzled-related protein) family members and WIF1 (Wnt inhibitory factor). To me, cleavage is most likely to affect the ability of Wnt5a and b to bind and/or activate their Frizzled receptors and LRP5/6 co-receptors. If cleavage indeed occurs within the minimal MMP-3 binding domain, than processed Wnt5a and b would lack a substantial part of their C-terminus. Pertinent here, similar carboxy-terminal deletions in murine Wnt1 and *Xenopus* Wnt8 have been reported to have dominant-negative activity and were shown to strongly inhibit embryonic responses to Wnt signaling (Hoppler *et al.*, 1996). All these possibilities will be tested once I know the cleavage sites and have obtained specific antibodies against Wnt5a and b.
- DNA microarray analysis has successfully been used to identify genes regulated by Wnt5a in C57mg cells (Prieve and Moon, 2003) and Wnt5b in 3T3-L1 preadipocytes (Kanazawa *et al.*, 2005). I will also use this technology to monitor and compare potential changes in gene expression induced by full length and processed Wnt5a and b. Interestingly, MMP-3 was almost four fold upregulated by Wnt5a in C57mg cells, suggesting the possible existence of a negative feedback loop. Eldar *et al.* (2003) proposed that such self-enhanced ligand degradation might represent a general mechanism for ensuring the robustness of long-range morphogen gradients.

Task 3b: in progress. I originally proposed to use the TVA system developed by the Varmus lab to determine a role for both the full length and processed forms of Wnt5a and b during mammary gland development and/or carcinogenesis. This elegant system takes advantage of the fact that mammalian cells are resistant to retroviral infection by subgroup A avian sarcoma leucosis viruses (ASLVs), but can be rendered susceptible by cell/tissue specific expression of the *tv-a* gene encoding the avian cell surface receptor for ASLV. Although Fisher *et al.* (1999) reported the generation of MMTV- and WAP-*tv-a* mouse lines to enable gene delivery in the mammary gland, research studies using these transgenic mice have yet to be published. The success rate of infection greatly depends on the route of viral delivery. Direct injection of concentrated virus into an organ of interest has been shown to be the most efficient infection method *in vivo*. This would require injection into the nipple without penetrating the ductal network in order to obtain efficient transduction of the mammary epithelium, which is technically very difficult. Furthermore only pubertal or pregnant mice can be used as recipients, as target cells must be actively replicating for successful infection. Primary MMTV/WAP-*tv-a* epithelial cells can be infected *in vitro*, but need to be transplanted into surgically cleared mammary fat pads of immunocompromised *scid/scid* mice, which display reduced outgrowth potential presumably due to altered hormone levels (M. Sternlicht, personal communication). Other limitations of this approach include 1) the relatively small 2.5 kb

insert capacity of the Rous Sarcoma Virus derived replication-incompetent ASLV vector RCAS and 2) the fact that the MMTV and WAP promoters driving *tv-a* are preferentially expressed in differentiated cells, rather than stem cells which are thought to be the etiological cell type of breast cancer. Most likely all or a combination of the above described limitations are responsible for the apparent lack of publications with the MMTV/WAP *tv-a* transgenic animals. More recently, Welm *et al.* (2005) developed an alternative mouse stem cell virus based system that involves retroviral delivery of genes into primary mammary epithelial cells, followed by transplantation of the transduced cells into cleared inguinal mammary fat pads. One advantage of this approach is that the retrovirus can target multiple cell types of the breast, including mammary progenitor cells. Another advantage is that commercially available syngeneic mice can be used instead of transgenic donor mice and *scid/scid* recipient mice. Unfortunately the success rate of this method greatly depends on the transgene used. Although it is a useful model to study the effect of proto-oncogenes on tumor development in the breast, transduced ducts are poorly represented in transplants if the transgene fails to confer a 'growth advantage'. For example, roughly two-thirds of the ducts in a reconstituted gland are transduced when a retrovirus is used containing the proto-oncogene *MYC*, while only one transduced (green) duct can be observed in a total of ten transplants with a GFP only retrovirus (A. Welm, personal communication). I tried to further optimize this approach by using selectable markers such as puromycin and blasticidin to enrich for infected cells, but failed to significantly increase the number of transduced ducts per reconstituted gland (data not shown). After unsuccessfully trying the method of Welm *et al.* (2005) with the MMP-3 retrovirus described in Task 2a, I decided together with colleague Dr. Bryan Welm to develop a novel viral system to aid gene function studies in the mammary gland. This system is lentiviral based in order to efficiently infect the non-dividing or slow dividing mammary progenitor cells *in vitro* and will be inducible for both gene-expression and knock-down, so that the function of a gene can be analyzed even if its manipulation negatively affects mammary gland development and/or tumorigenesis.

I Viral design: in progress. The final design of the expression vectors required extensive cloning and testing. The RNA interference vectors are still under construction, but their design is based on a modification of existing viruses. The self-inactivating feature of the transfer vector allows for introduction of a new promoter to drive expression of the transgene. Several constitutive promoters were tested for their strength and robustness in the mammary gland, including CMV, PGK, CAG and EF1α. The latter promoter was found to be the strongest and most stable without negatively affecting viral titers. The PGK promoter was chosen for constitutive expression of a fluorescent marker downstream of the inducible TRE-tight promoter, since it is relatively small and does not enhance background expression from the TRE-tight promoter. We also compared several fluorescent markers and found ZsGreen to be twice as bright as eGFP, while dTomato is brighter and easier to identify by FACS than H2BmRFP. Some researchers insert WPRE (woodchuck posttranscriptional regulatory element) between the 3' end of the transgene and the 3'LTR of the transfer vector to improve the performance of their retroviral and lentiviral vectors (Zufferey *et al.*, 1999). We left this element out in our vectors as it did not improve viral titers and even lowered the expression of the transgene when used in combination with an IRES (internal ribosomal entry site). We further found that the

promoter driving the transgene needs to be cloned in the same (sense) orientation as the viral 5'LTR, as the anti-sense orientation will greatly reduce the viral titer due to the formation of double stranded RNA. This is even true for the TRE-tight promoter, which is not supposed to be active in our packaging cell line HEK 293T.

The transfer vectors that we intend to use for this study are depicted in Figure 8 and are derivatives of the self-inactivating lentivirus pSICO (Ventura *et al.*, 2004). I Constitutive expression: the EF1α promoter will constitutively express a transgene as well as a fluorescent marker, either Zsgreen as depicted in Figure 8 or dTomato, so that transduced ducts can easily be identified in the reconstituted glands. II Inducible expression: inducible expression of a transgene requires co-infection of two viruses containing different fluorescent markers. One vector will constitutively express the improved reverse tetracycline transactivator rtTAS2-M2 (Urlinger *et al.*, 2000) together with Zsgreen from the EF1α promoter. The other vector will constitutively express dTomato from the PGK promoter, while the transgene is under the control of the Tet-responsive TRE-tight promoter. Addition of doxycycline to the drinking water and/or food of the mice promotes binding of rtTAS2-M2 to the TRE-tight promoter and consequently expression of the transgene. The efficiency of co-infection *in vitro* is at least 90% and double transduced ducts are both green and red. III Constitutive knock-down: A small interfering ds RNA (siRNA) is expressed from the human U6 promoter, while the EF1α promoter drives a fluorescent protein to mark the transduced duct. IV Inducible knock-down: inducible knock-down of a gene also requires co-infection of two viruses containing different fluorescent markers. One vector is a modification of pSICO; the CMV eGFP stuffer that resides between the two lox sites and prevents expression of the siRNA has been replaced with the EF1α promoter driving the expression of both rtTAS2-M2 and ZsGreen. The other vector is the TRE-tight virus described above containing the CRE-recombinase. Addition of doxycycline to the drinking water and/or food of the mice promotes expression of the CRE-recombinase, which excises the stuffer and enables expression of the siRNA. Thus double transduced ducts are both green and red, but gene knock-down is accompanied by a loss of green fluorescence.

II Viral production: completed. We have tried and optimized two different lentiviral packaging systems. The 'second generation' three plasmid system described by Naldini *et al.* (1996) uses in addition to the transfer vector the packaging construct pCMVΔR8.9 and a plasmid containing a heterologous envelope protein, such as the G glycoprotein of vesicular stomatitis virus (VSV-G). We no longer use this system as pCMVΔR8.9 is difficult to amplify in *E. coli* and large quantities of DNA are required for introduction of these plasmids in HEK 293T cells by the (inexpensive) calcium-phosphate transfection method. The 'third generation' four plasmid system described by Dull *et al.* (1998) works equally well, gives high DNA yields and provides increased biosafety as it employs a minimal set of HIV genes on two separate packaging plasmids. We routinely get titers of 10^7 to 10^8 transducing units [TU]/ml with this method after concentration and storage at -80°C.

III MEC isolation, culturing and infection: completed. We essentially use the same MEC isolation and culturing protocol as Welm *et al.* (2005), but developed a non-FACS based

method to enrich for cells with enhanced outgrowth potential (Figure 9). These cells can be infected efficiently with both retro- and lentiviruses after this procedure: we routinely get 80% infected without using a selectable marker to enrich for transduced cells.

IV Results: in progress. Figure 10 shows a clear difference in efficiency between the Welm *et al.* (2005) method and our approach when using a growth neutral/fluorescent marker only virus. We performed a large number of surgeries using cells infected with several different viruses, including viruses expressing MMP-3, Wnt5a and Wnt5b, and are currently waiting for these transplants to grow out.

Our system should greatly benefit the mammary gland research community, as it allows one to relatively quickly assess the role of a large number of genes and their interactions during mammary gland development and tumorigenesis, without having to make costly and time-consuming transgenic animals. We anticipate submitting a manuscript describing this technique before the end of this year.

Key Research Accomplishments

- The hemopexin domain of MMP-3 can interact with the C-terminal portion of Wnt5a and b in a yeast two hybrid assay
- The catalytic domain of MMP-3 can cleave i) cell bound Wnt5a and b immunoprecipitated from RIPA cell lysates and ii) soluble Wnt5b, but not soluble Wnt5a
- MMP-3 and Wnt5b are both expressed in the ductal microenvironment during mammary gland development
- Development of a lentiviral based method to aid gene function studies in the mammary gland

Reportable Outcomes

None to date

Conclusions

The yeast two-hybrid system was used to identify MMP interacting proteins as potential novel substrates. Only the hemopexin domain appeared suitable as 'bait', since the catalytic domain is 'sticky' and full length MMPs are poorly folded in the yeast cytoplasm. The hemopexin domain of MMP-3 interacted with the C-terminus of Wnt5b in a two-hybrid assay. A deletional analysis of this clone revealed a minimal MMP-3 binding domain of 55 amino acids, which is flanked by cysteine residues and is one of the least conserved domains among the 19 mouse Wnts. A hinge/blade swapping approach with the hemopexin domain of MMP-10 further revealed that the hinge region and 3 of the 4 MMP-3 hemopexin domain blades are required for binding of the Wnt5b C-

terminus. The C-terminus of Wnt5a is 84% identical to Wnt5b and is the only other Wnt expressed in the mammary gland that interacts with MMP-3 in the two-hybrid assay. The C-termini of Wnt2, 4, 6, 7b and 10b did also not interact with the hemopexin domains of MMP-2 and MMP-14. Collectively, these two-hybrid observations suggest that the interaction between MMP-3 and Wnt5a and b is quite specific. Recombinant MMP-3 catalytic domain can cleave both soluble Wnt5b as well as cell bound Wnt5a and b immunoprecipated from RIPA cell lysates. Cleavage seems to occur within the minimal MMP-3 interacting domain as judged by mobility shift on SDS-PAGE and can be blocked by the MMP inhibitors GM-6001 and EDTA. These observations suggest that both Wnt5a and b are MMP-3 substrates, but only Wnt5b is co-expressed with MMP-3 in the ductal microenvironment during mammary gland development. A novel lentiviral based method is currently being developed to analyze the role of Wnt5a and b and their MMP-3 cleavage products during mammary gland development and/or carcinogenesis.

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Table 1: Outcome of the two-hybrid screens

- mM3-CD interacts with 1 out of ± 500 clones

- mM3-FL: 9.8×10^5 transformants screened
  57 true positives
  33 proper cDNA fusions
  8 different intracellular proteins

-mM3-HPX: 7.4×10^5 transformants screened
  98 true positives
  58 proper cDNA fusions
  19 different intracellular proteins
  → **2 extracellular proteins: last 147 aa of Wnt5b**
  last 280 aa of Reprolysin/ADAM-TS18

- hM3-CD interacts with 1 out of ± 1000 clones

- hM3-FL: 7.5×10^5 transformants screened
  326 putative candidates
  to many to pursue

- hM3-HPX: 1.4×10^6 transformants screened
  5 true positives
  5 proper cDNA fusions
  2 different intracellular proteins

Table 2: Yeast two-hybrid interactions between Stromelysin-1 and Wnt5b

	lexA DB	mM3-HPX	mM3-FL	mM3-CD
Gal4 AD	-	-	-	-
Wnt5b 147 aa	-	++	-	+++
Wnt5b FL	-	-	-	+

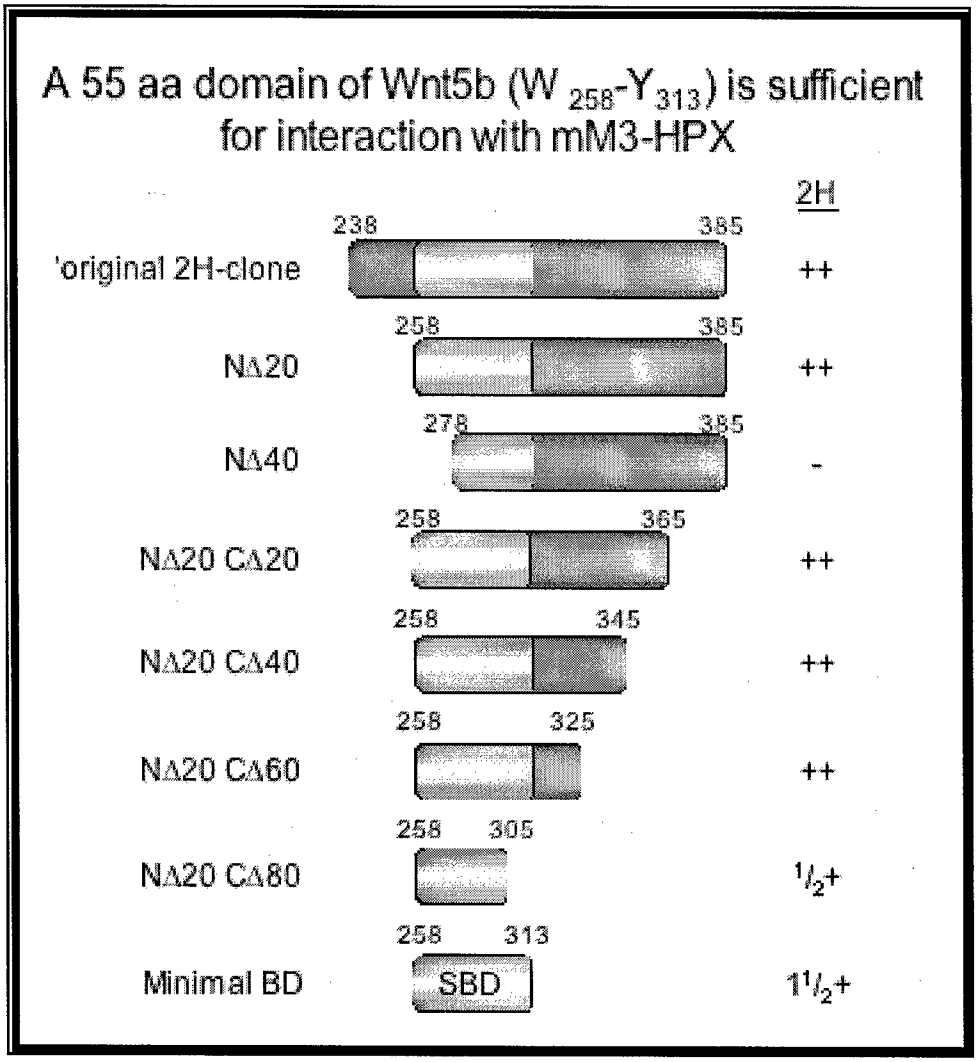

Figure 1. Identification of a minimal MMP-3 hemopexin binding domain within Wnt5b

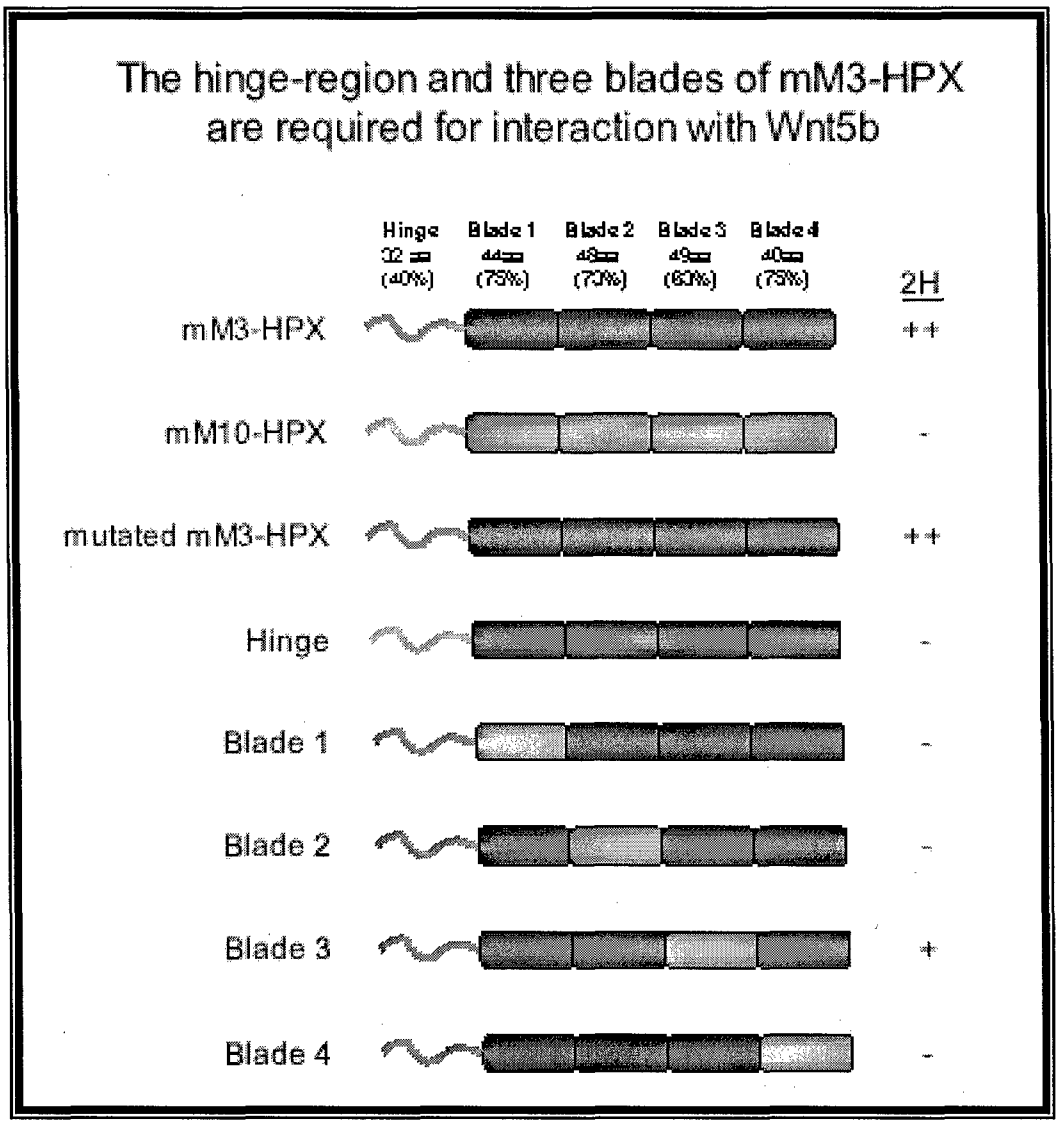

Figure 2. Identification of the domains within mM3-HPX necessary for the interaction with the C-terminus of Wnt5b. Mutated mM3-HPX contains several silent point mutations, which created unique restriction sites and enabled the domain swapping with mM10-HPX.

Table 3: Yeast two-hybrid interactions between MMP hemopexin domains and Wnt's

	lexA DB	MMP2	MMP3	MMP10	MMP14
Gal 4 AD	-	-	-	-	-
Wnt2 160 aa (55%)	-	-	-	-	-
Wnt4 151 aa (52%)	-	-	-	-	-
Wnt5a 147 aa (84%)	-	-	++	-	-
Wnt5b 147 aa (100%)	-	-	++	-	-
Wnt6 148 aa (47%)	-	-	-	-	-
Wnt7b 154 aa (49%)	-	-	-	-	-
Wnt10b 147aa (45%)	-	-	-	-	-

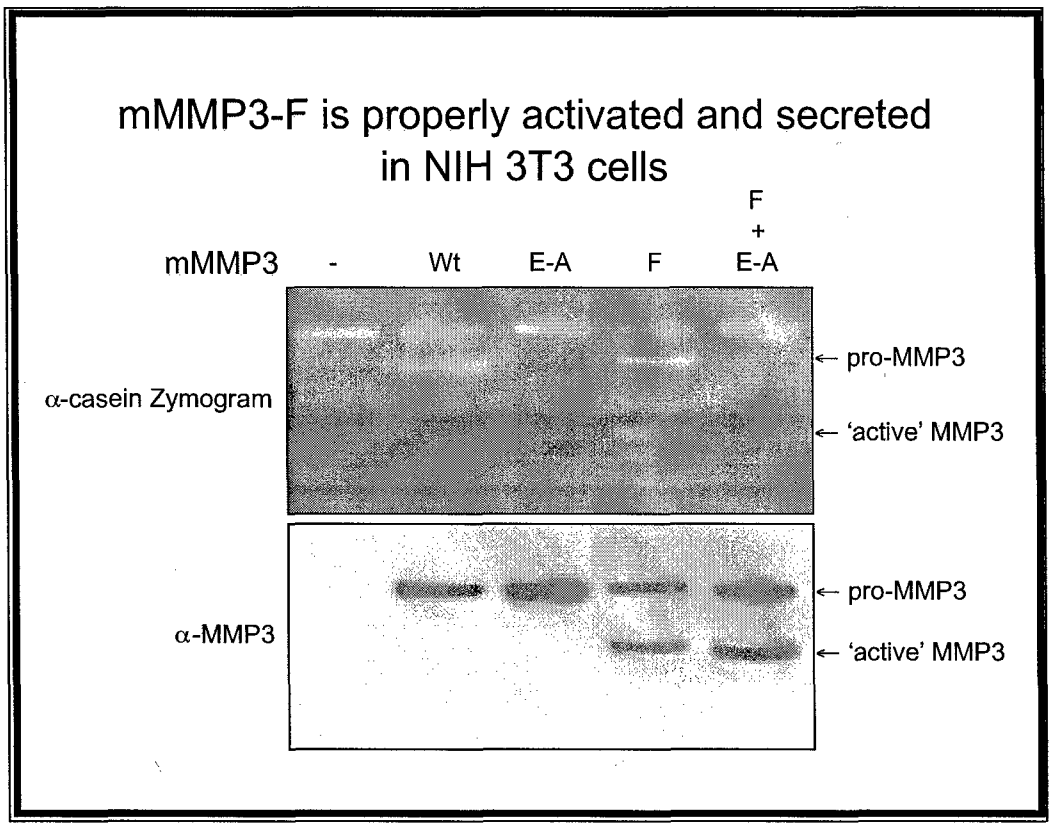

Figure 3. The furin activatable version of mMMP-3 is properly activated and secreted in NIH 3T3 cells. NIH 3T3 cells infected with either an empty retrovirus (-) or a retrovirus expressing wild type (Wt), catalytically dead (E-A), furin activatable (F) or furin activatable but catalytically dead (F + E-A) version of mMMP-3 were overlaid with serum free media for 24 hrs and their culture supernatant was subsequently filtered and concentrated. The equivalent of 10 µg of medium proteins from each of these samples were analyzed by both α-casein zymography and anti-MMP-3 Western blotting.

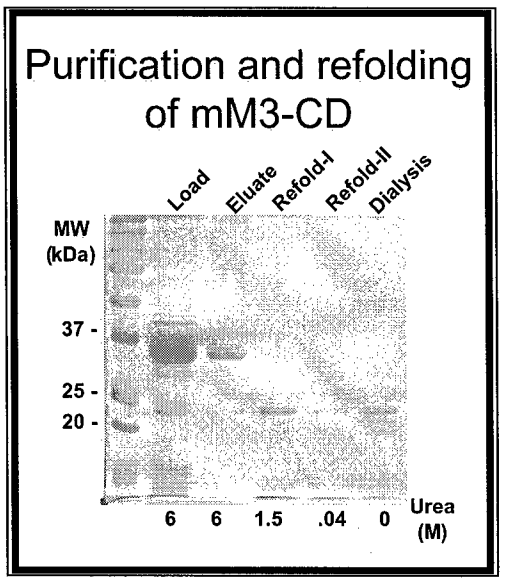

Figure 4. Purification and refolding of recombinant murine MMP-3 catalytic domain. A N-terminal 6xHis tagged version of the catalytic domain with its inhibitory pro-domain was expressed in *E-coli* and solubilized from inclusion bodies with 6M urea. This material was applied to a Ni-column ('Load') and mM3-CD was eluted with 250mM imidazole ('Eluate'). mM3-CD was refolded by drop-wise dilution to a final protein concentration of 100 µg/ml into refolding buffer with 1M urea ('Refold-I'), followed by drop-wise dilution into refolding buffer without urea ('Refold-II'). Finally, mM3-CD was dialyzed to remove any remaining urea and imidazole ('Dialysis').

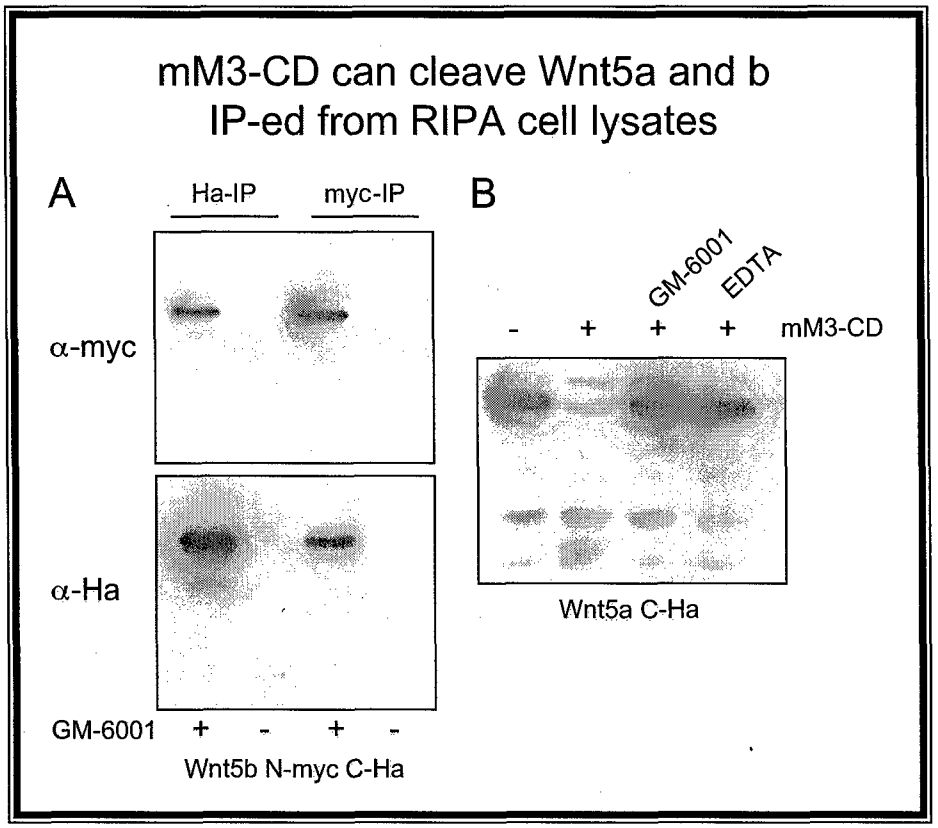

Figure 5. Recombinant murine MMP-3 catalytic domain can cleave Wnt5a and b immunoprecipitated from RIPA cell lysates. NIH 3T3 cells infected with a retrovirus expressing either double tagged Wnt5b (A) or single tagged Wnt5a (B) were collected in RIPA lysis buffer and 500 µg of total protein was used per IP with agarose immobilized anti-HA or anti-myc. Each IP was subsequently incubated O/N with 1 µg of AMPA activated mM3-CD in the absence or presence of either 10µM GM-6001 or 1mM EDTA and analyzed by Western immunnoblotting.

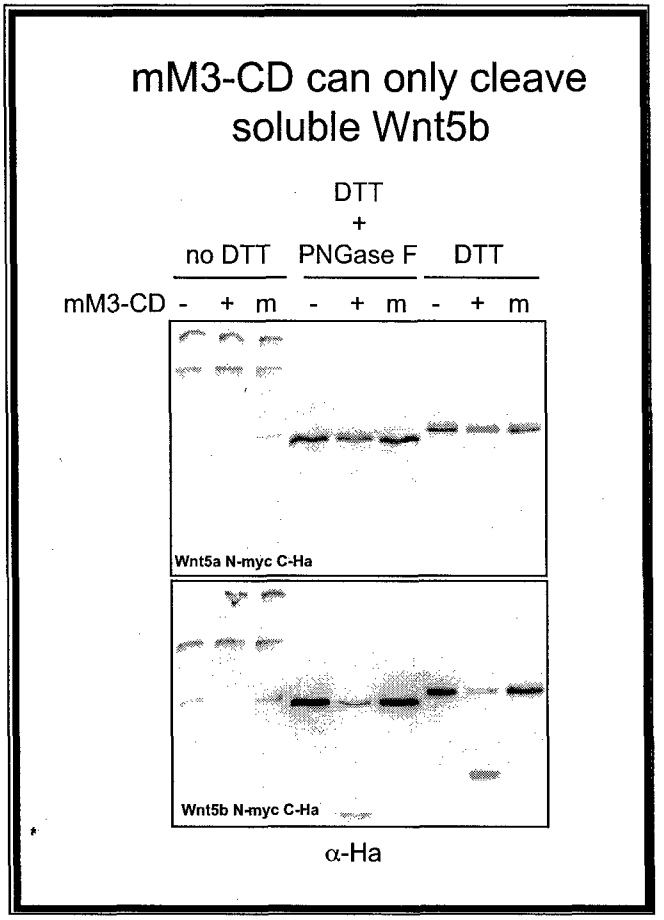

Figure 6. Recombinant murine MMP-3 catalytic domain can only cleave soluble Wnt5b. NIH 3T3 cells infected with a retrovirus expressing double tagged Wnt5a or b were overlaid with serum free media for 24 hrs and their culture supernatant was subsequently filtered and concentrated. The equivalent of 100 µg of medium proteins was either kept on ice (-) or incubated at 37°C for 4 hrs in the presence (+) or absence (m) of 1 µg AMPA activated mM3-CD. Each of these samples was than split in three to asses the mobility of Wnt5a or b after Peptide:N-Glycosidase F treatment and boiling in SDS-PAGE sample buffer with or without the reducing agent DTT. Samples were analyzed by anti-Ha Western blotting.

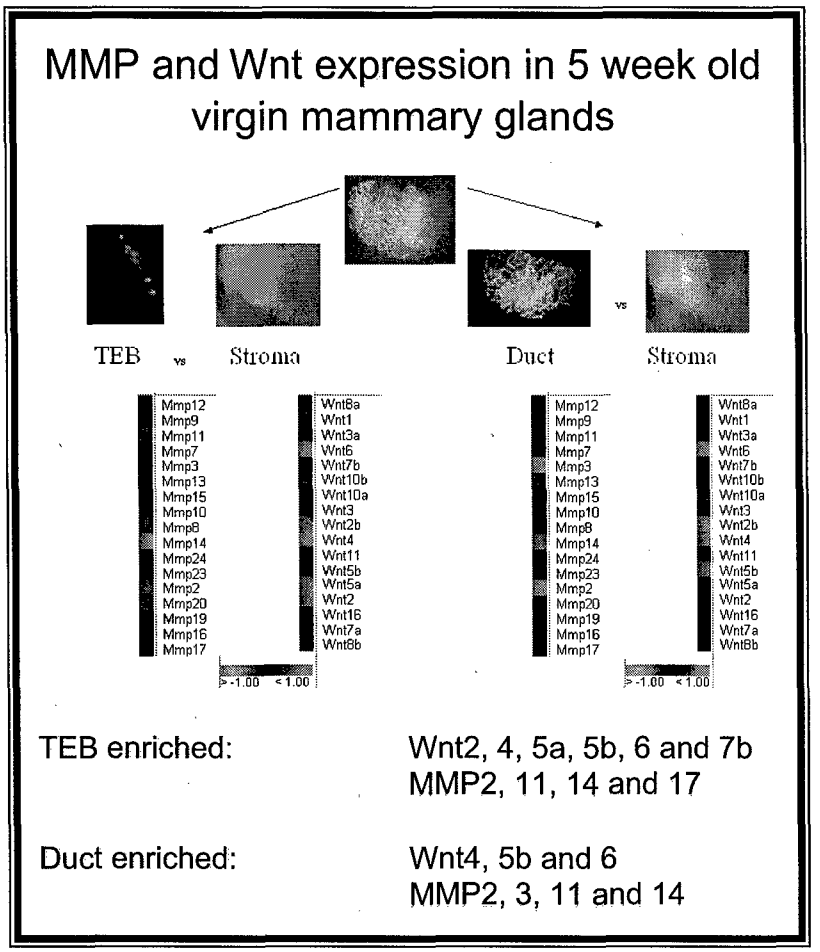

Figure 7. Identification of MMP and Wnt transcripts that are specifically upregulated in the different structures of the mammary gland. Terminal end buds (TEBs) and ducts were dissected from mammary glands of five week old virgin CAG-eGFP mice.Their transcriptional profile was compared with that from distant stroma using microarray technology.

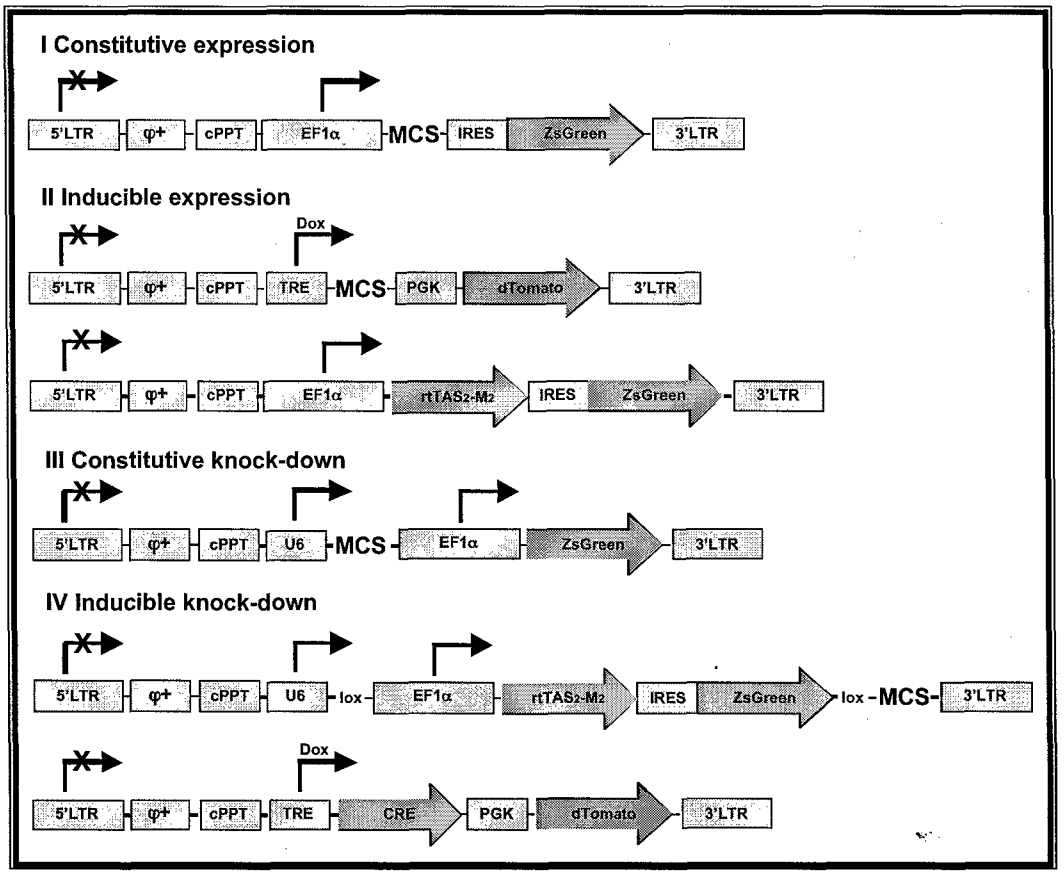

Figure 8. Lentiviral transfer vectors used in this study. 5'LTR; 5' long terminal repeat. φ+; extended packaging signal, includes RRE (Rev-response element) which enables nucleo-cytoplasmic transport of the viral mRNA. cPPT; central polypurine tract, improves nuclear translocation of the pre-integration complex. 3'LTR; 3' long terminal repeat, provides the self-inactivating feature of the vector as it contains a deletion in the U3 enhancer region. All these elements are depicted in yellow and were derived of pSICO (Ventura *et al.*, 2004). See text for an explanation of the other elements.

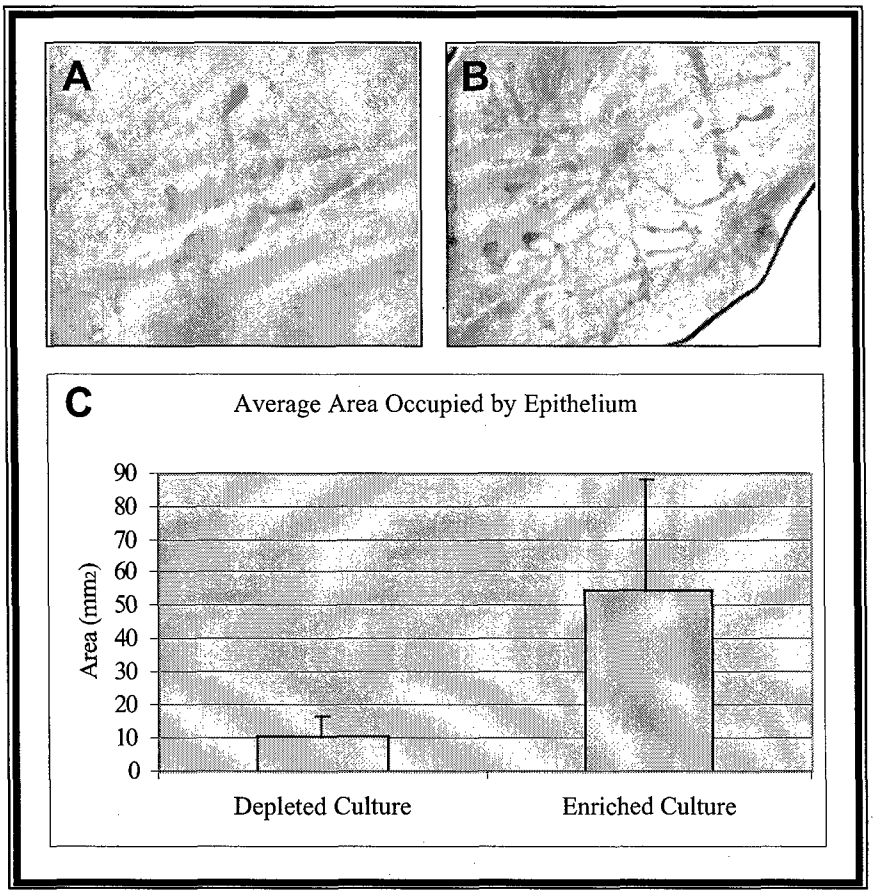

Figure 9. Cultured primary MECs can be enriched for increased outgrowth capacity. The inguinal mammary glands of a three week old virgin were cleared and injected with 50.000 primary MECs that were either depleted (A) or enriched (B) for cells with enhanced outgrowth capacity. The reconstituted glands were visualized by carmine alum staining four weeks after transplantation. The average area covered by the transplanted epithelium is quantitated in (C). Four transplants per culture condition were measured and the error bars indicate a standard deviation.

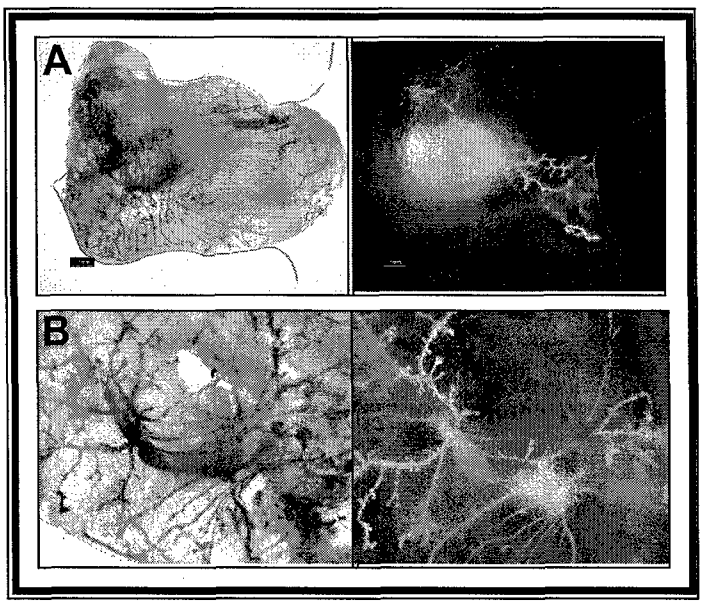

Figure 10. Representative transplants generated by the method of Welm *et al.* (2005) (**A**) and our novel lentiviral based approach (**B**). More transduced ducts are present in a reconstituted gland when primary MECs are infected with our approach compared to the method of Welm *et al.*(2005).

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WNT5B IS AN IN VITRO SUBSTRATE FOR STROMELYSIN-1

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Matrix metalloproteinases (MMPs) are a family of zinc-dependent extracellular endopeptidases that participate in both physiological and pathological processes. They have traditionally been viewed as effectors of late stages of cancer evolution, as they are positive regulators of angiogenesis and facilitate invasion and metastasis by degrading structural extracellular matrix components. However, Sternlicht et al. (1999) showed that targeted expression of autoactivated rat Stromelysin-1 in mouse mammary glands is sufficient to induce hyperplasias and breast cancer, demonstrating that this MMP can act as a natural tumor promoter. To understand how MMP-3 can influence cancer susceptibility, the yeast two-hybrid system was used to identify interacting proteins as potential novel substrates.

The hemopexin domain of MMP-3 interacted with the C-terminus of Wnt5b in a two-hybrid assay. A deletional analysis of this clone revealed a minimal MMP-3 binding domain of 55 amino acids, which is flanked by cysteine residues and is one of the least conserved domains among the 19 mouse Wnts. A hinge/blade swapping approach with the hemopexin domain of MMP-10 further revealed that the hinge region and 3 of the 4 MMP-3 hemopexin domain blades are required for binding of the Wnt5b C-terminus. The C-terminus of Wnt5a is 84% identical to Wnt5b and is the only other Wnt expressed in the mammary gland that interacts with MMP-3 in the two-hybrid assay. The C-termini of Wnt2, 4, 6, 7b and 10b did also not interact with the hemopexin domains of MMP-2 and MMP-14. Collectively, these two-hybrid observations suggest that the interaction between MMP-3 and Wnt5a and b is quite specific. Recombinant MMP-3 catalytic domain can cleave both soluble Wnt5b as well as cell bound Wnt5a and b immunoprecipitated from RIPA cell lysates. Cleavage seems to occur within the minimal MMP-3 interacting domain as judged by mobility shift on SDS-PAGE and can be blocked by the MMP inhibitors GM-6001 and EDTA. Our current efforts focus on identifying the cleavage site within Wnt5a and b and to try demonstrate MMP-3 mediated cleavage of these molecules in vivo. Furthermore, we are developing an in vitro assay to measure the effect of cleavage on Wnt signaling.

Although Wnts have diverse roles during development, they seem to function in homeostasis in adults and inappropriate activation of the Wnt signaling pathway(s) has been implicated in a variety of cancers, including breast cancer. Wnt proteolysis by MMPs would constitute a novel way of modulating the activity of these growth factors. The role of Wnt5a and b in the mammary gland is still not well understood. It will be

interesting to see whether inappropriate proteolysis of these molecules by MMP-3 contributes to breast cancer induction and/or progression.

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