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Concept Award: Discovery of novel mammary developmental and cancer genes using ENU mutagenesis.

Christopher Ormandy, Garvan Institute of Medical Research, Sydney, and Christopher Goodnow, John Curtin School of Medical Research, Australian National University, Canberra.

Introduction

To date there has been no systematic search for genes controlling mammary development and carcinogenesis, and this project seeks to undertake the first such search by participation in the large scale recessive ENU mutagenesis project currently being conducted by Prof. Chris Goodnow, at the John Curtin School, Australian National University (ANU).

Functional characterisation of genetic loci is urgently required if the imminent completion of the human and mouse genomes is to be of immediate benefit. This requirement is being met in a number of ways, including seven large scale ENU mutagenesis projects under way worldwide. Of these, just three are undertaking genome wide recessive screens. Recessive mutations, requiring the loss of function of two alleles, provide far greater insight into the genetic control of normal development and carcinogenesis than dominant, gain-of-function mutations, but require more complex breeding of pedigrees. This has been achieved at the ANU, where 200 G3 gender balanced pedigrees of 25 animals have been produced and aged to 8 months. On average each pedigree carries 100 defective genes, every animal carries 12 homozygous functional mutations, and 3 animals per pedigree will carry null mutations for the same gene. Thus approximately 20 000 homozygous null mutants have been produced; more than 20% of the mouse genome has been scanned. Litter size has fallen from 8 in wild type animals to 2.3 in the G3 generation, indicative of lethal recessive mutations. A preliminary blood screen using multi parameter FACS analysis and a paediatric blood auto analyser revealed 10 strains with lymphocyte disorders and 2 with platelet abnormalities. Thus the mutagenesis regime employed has been effective and sufficient numbers of G3 animals have been obtained.

Screening was undertaken using whole mount histology of the 4^{th} inguinal mammary gland. Approximately 1100 mice were analysed, and a number of abnormalities and tumors were identified. From these 6 pedigrees were selected for propagation. Currently members of these pedigrees are being tested by mammary biopsy to identify affected individuals for use in mapping crosses. This award allowed the initial screening to proceed and for the breeding of the 6 selected pedigrees. The successful screening has ensured that the project has continued to attract funding, and is now funded (from the end of 2001) to proceed for the next 3 years.

Body

The research undertaken with this award consisted of the following steps;

1. Estimation of the natural variation and tumor incidence in the mammary ductal structure of aged virgin animals of the C57Bl6 strain.

2. Screening of approximately 1100 mice from the pedigrees produced by the first round of ENU mutagenesis and identification of pedigrees containing multiple individuals with mammary defects.

3. Regeneration and aging of selected pedigrees with mammary tumors from genetic back-ups to confirm the genetic basis of the observed defects.

4. (currently ongoing) Biopsy testing of female progeny from the regenerated pedigrees to identify affected individuals for breeding to produce homozygous breeding lines.

1. To interpret the screening results we needed to estimate the rate at which mammary defects and tumors occurred spontaneously in the C57Bl6 animals used for the ENU mutagenesis program. We collected 30 mammary glands from virgin females aged between 15 and 40 weeks. Whole mount analysis showed a consistently poorly side branched ductal system with very little increase in ductal side branching (IDSB) with age. No tumors were seen and only 2 individuals with IDSB were seen. This work estimated the natural frequency of IDSB to be 67 in 1000.

2. Results of the screen are presented in Figure 1. The most common abnormality observed was IDSB, with 25% (50 of 200) of pedigrees showing at least 1 affected individual. Invasive lesions were classified as single or multiple and overall 11 pedigrees were found with an example of multiple invasive lesions. The association of these phenotypes was examined by removing the remaining glands (left and right gland number 2 and 3, and the remaining number 4) from every individual found with an abnormality (Figure 2). Increased ductal side branching was seen in 108 glands and appeared to be a predictor of invasive lesions as 75% of multiple invasive lesions (n=32) and 56% (n=34) of single invasive lesions appeared in highly side branched glands. The co-occurrence of a tumor (single or multiple) and IDSB by chance is estimated at 0.6% if independent, but is observed in 4.8% of affected glands. The situation is more extreme for multiple tumors where 0.03% of affected glands should show both IDSB and MIL by chance, but 2.5% are observed.

We chose 6 pedigrees to pursue, based on the nature of the defect and available resources. The details of each are shown in Figure 3. These pedigrees all show increased ductal side branching in addition to a second lesion, mostly multiple invasive lesions or ductal carcinoma in situ. When we examined the remaining glands from an affected animal we generally observed the same phenotype, arguing against a chance occurrence (Figure 4).

3. Breeding of genetic backups.
The brothers of the female used in the first screen were used to recreate each pedigree. Multiple males were used given the 1 in 2 chance that they carried the mutation. Each pedigree was recreated using an inbreeding scheme, where female progeny of father daughter matings were aged for screening.

4. Biopsy testing.

The essential difficulty here is that tumors may not develop until after the animal has passed its useful breeding age. To overcome this problem we have relied on the association between tumors and increased ductal side branching, reasoning that if this is a precursor lesion then it will be apparent at an earlier age. We have also examined the glands very carefully as tumors may be very small. Approximately 30% of the animals were biopsied at 5-6 months of age. Increased ductal side branching has been found, and one animal showed a small tumor. We expected to see more tumors than we found. As a consequence we will allow the next group to age to 6-7 months before biopsy. We will also biopsy an older group of animals that we originally intended to use simply to demonstrate regeneration of the initial phenotype. The original aim will be achieved and we will determine whether these old animals can be successfully mated.

Summary

A screen of the first round of large scale mutagenesis has been completed. Six pedigrees with members harbouring mammary tumors were identified. These pedigrees were recreated from genetic backups and are now being screened using mammary biopsy. Affected females will be used to establish stable breeding lines.

Key research accomplishments

• Screening of the first round of mutagenesis at 8 months of age and the identification of 6 pedigrees with mammary gland tumors.

• Recreation of these six pedigrees from male siblings of females screened in the first round using an inbreeding scheme.

• Biopsy screening of 1/3 of these animals to date at 5 months of age, with the identification of females showing increased ductal side branching and in one case a tumor. Future biopsy screening will use older animals, to increase tumor detection but at increased risk of failed breeding.

Reportable outcomes

• Poster presentation, Lorne Genome Conference, Feb 2001.

• Oral presentation, Victorian Breast Cancer Research Centre, Annual Meeting, March Melbourne 2001

• Oral presentation, Reproductive Genomics Meeting, Monash Medical Centre November Melbourne 2001

• Successful Funding DAMD17-1-01-0241 to continue this project from October 2001.

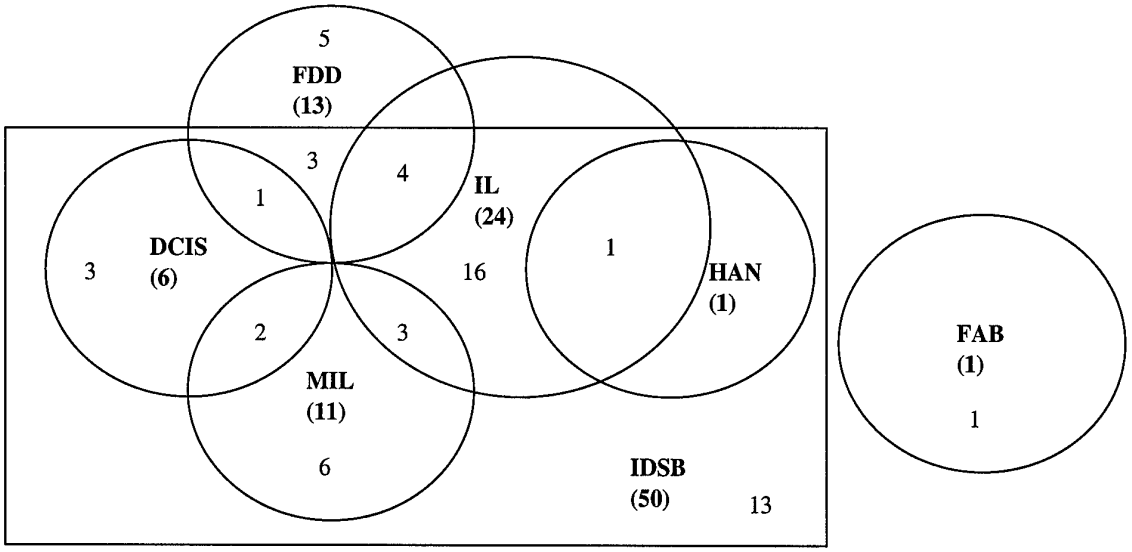
Conclusions

So What?

In the best case we have identified 6 mouse pedigrees harbouring null mutations causing cancer in mouse mammary glands, and have begun the mapping process to identify the responsible genes. If just one of these has relevance to human cancer it will represent a major advance in the field.

Appendices

Figures.

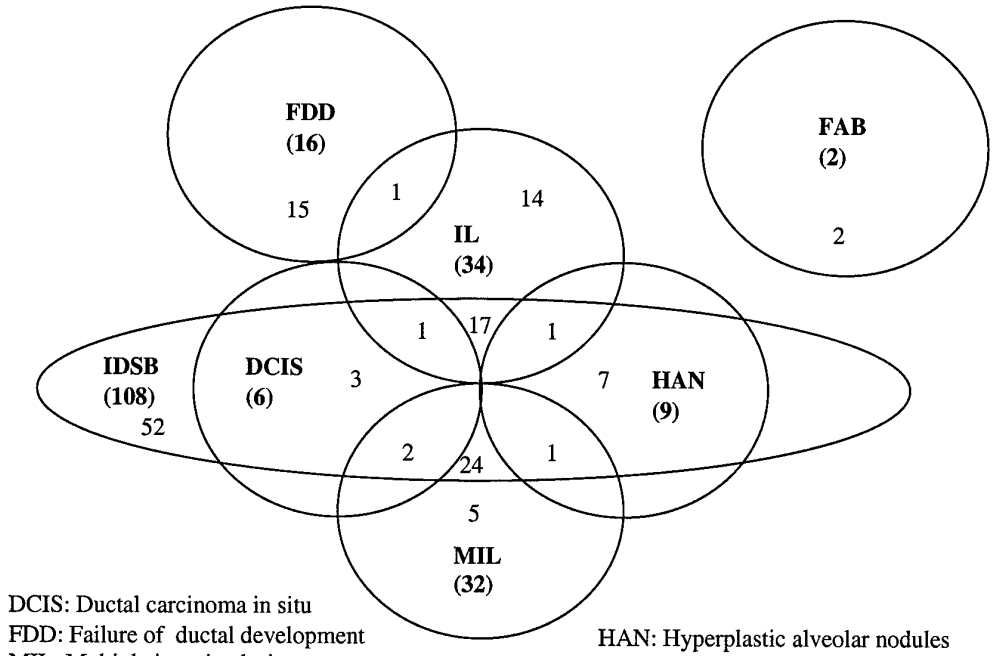


DCIS: Ductal carcinoma in situ
FDD: Failure of ductal development
MIL: Multiple invasive lesions
IL: Invasive lesion

HAN: Hyperplastic alveolar nodules
IDSB: Increased ductal side branching
FAB: Failure of alveolar bud development

Figure 1. Phenotype frequency

Number of pedigrees with individual(s) displaying defects.



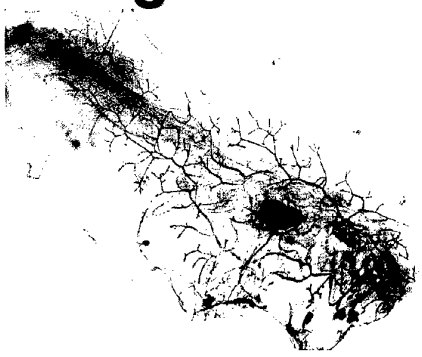
DCIS: Ductal carcinoma in situ
FDD: Failure of ductal development
MIL: Multiple invasive lesions
IL: Invasive lesion

HAN: Hyperplastic alveolar nodules
IDSB: Increased ductal side branching
FAB: Failure of alveolar bud development

Figure 2. Associated phenotypes

Number of glands with multiple phenotypes.

Pedigree 07



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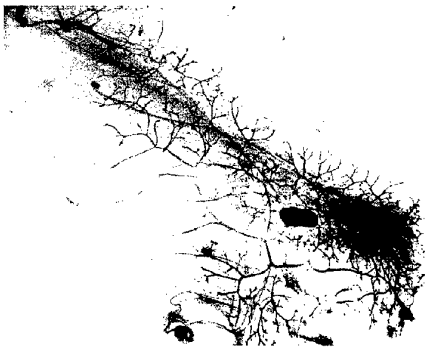

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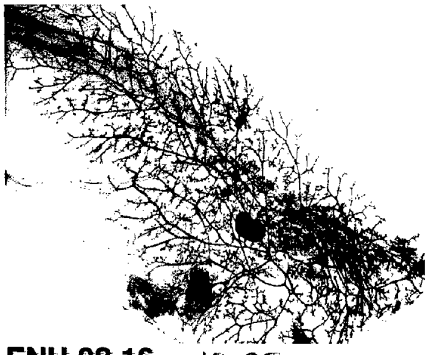
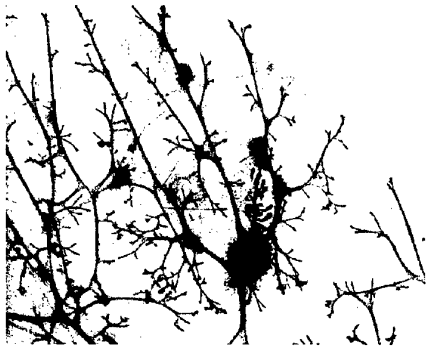
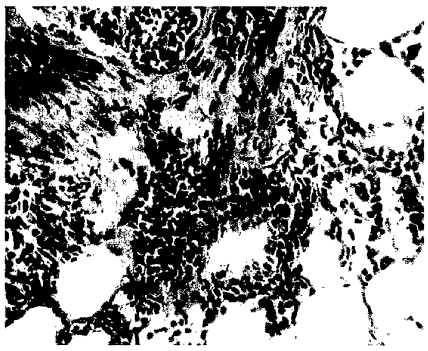
Pedigree 08







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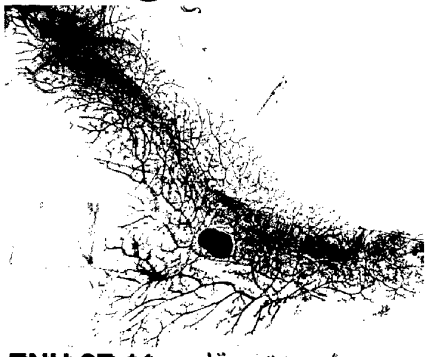
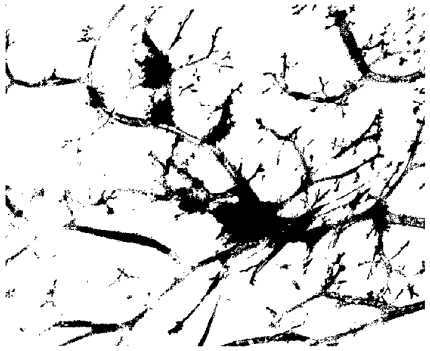
Pedigree 97





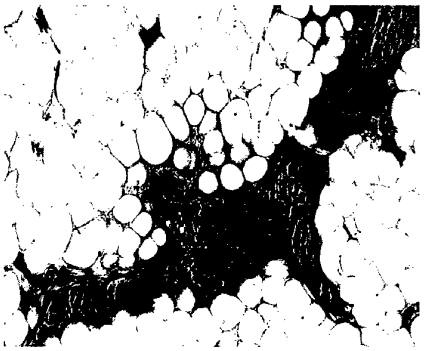


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Figure 3 continued

Pedigree 70

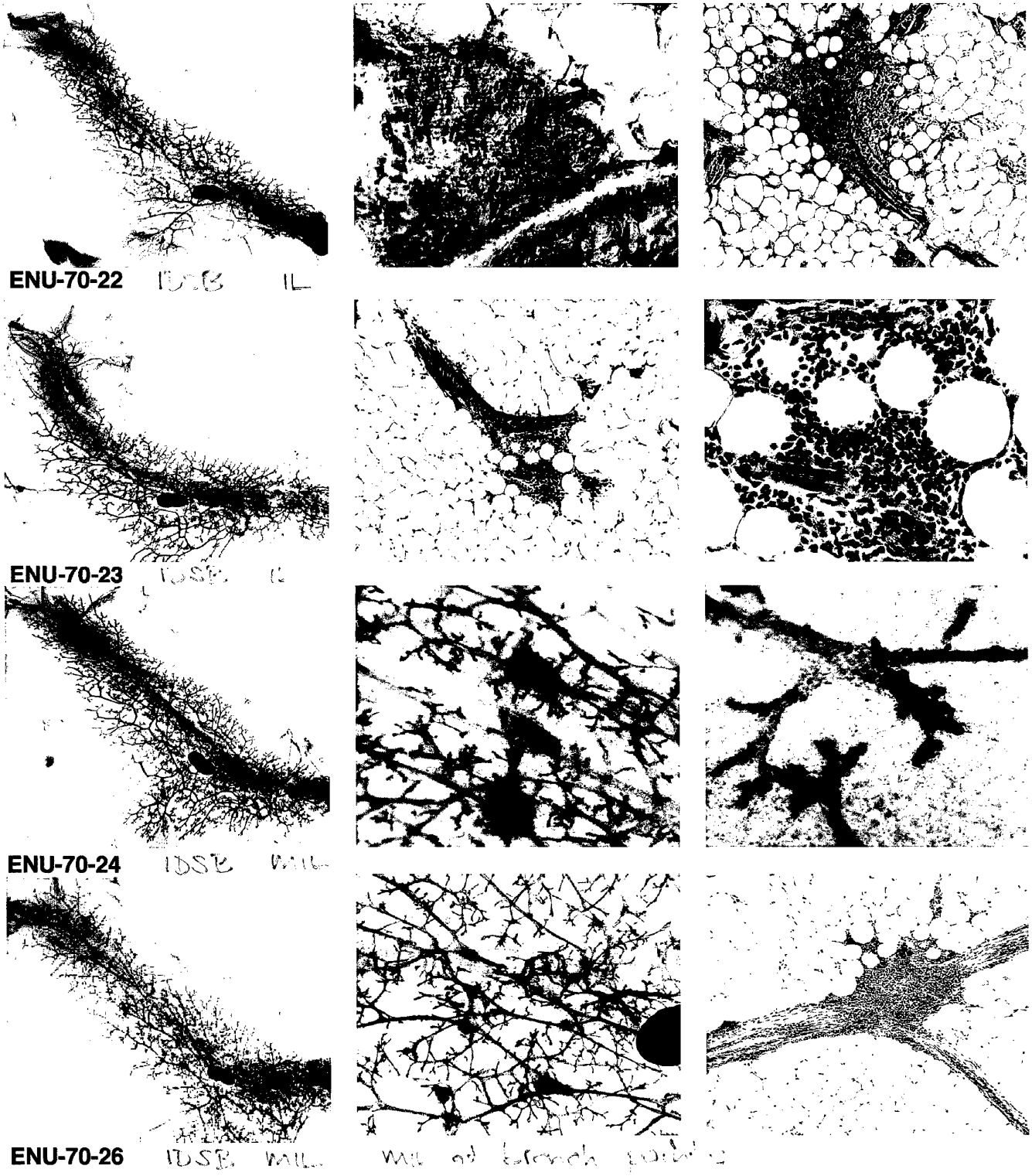
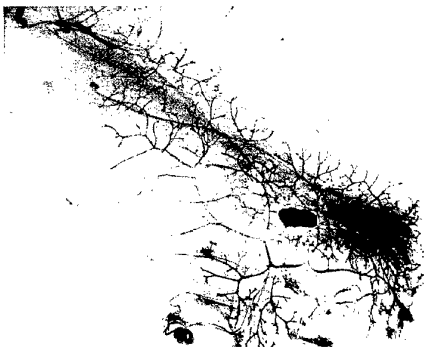
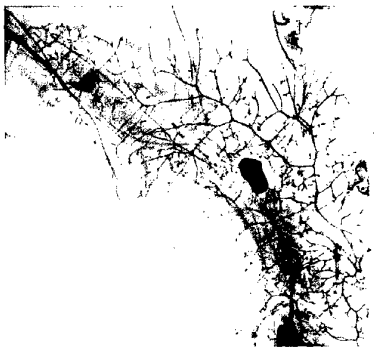
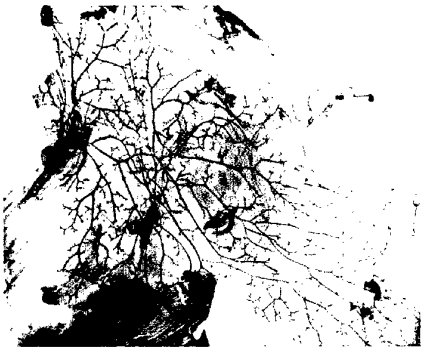


Figure 4







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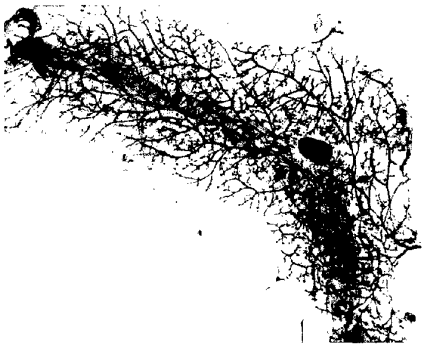

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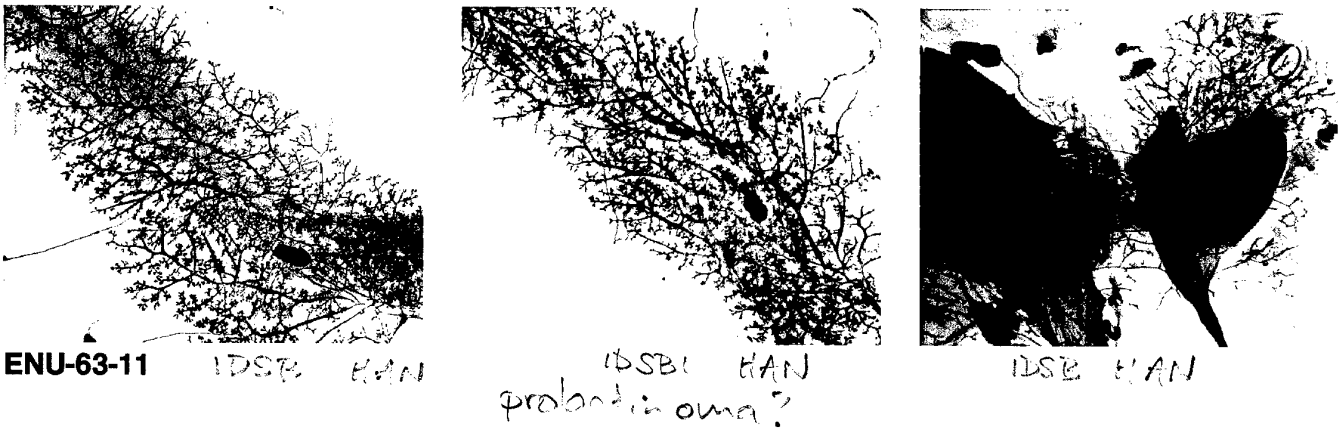

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