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6. AUTHOR(S)
Ellen Heber-Katz

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Wistar Institute
3601 Spruce Street
Philadelphia, PA 19104

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13. ABSTRACT (Maximum 200 words)

Our studies in the MRL mouse have shown that this mouse has unusual healing properties. This includes the closure of ear hole wounds which is not seen in any other mouse strain. This form of healing has many features that are similar to those seen in the regeneration of amphibian limbs. In amphibian models, it was shown that de-differentiation of mature cells contributes to the blastema and the growth of a new limb. It was of interest to determine if similar processes were at work in this mammalian model of regeneration. We explored several issues: 1) the ability of the regenerate environment to induce de-differentiation and re-differentiation of mature cells, 2) the ability of factors from the regenerate tissue to affect differentiation of cells in vitro, and 3) the induction of a blastema or events leading to blastema formation in a non-regenerating strain of mouse. Results in all of the experiments indicated that many of the same processes were in fact important in this mouse and its healing response.

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Goal: The goal of this seedling was to explore the regenerative potential of the blastema in the MRL mouse and to use that information to try to form a blastema in a non-regenerating mouse. The amphibian blastema has been shown to provide an environment which allows adult tissue to de-differentiate, proliferate, and re-differentiate into mature functional tissue. Since the MRL mouse forms a blastema and regenerates, we have examined its ability to effect a similar response in the growth and differentiation of muscle, nerve, cartilage, and bone of both mouse and human origin. The methodology involved 1) transplantation of these tissues into immuno-compromised MRL hosts and was assessed by histological and molecular criteria, 2) the examination of factors derived from the MRL for their effects in-vitro, and 3) the application of these principals to the formation of a blastema in a non-regenerating mouse.

Hypothesis: The MRL mouse will provide information to regenerate "nonregenerating tissue, mouse and human" both in-vivo and in-vitro.

Technical Approach: For the experiments carried out, we used 3 systems:

1. *In-vivo*: We injected mature cartilage into a growing MRL ear blastema
   a. MRL male tissue into MRL female ears
   b. C57BL/6 male tissue into C57BL/6 male ears (controls).
   c. Human tissue into MRL.Rag -/- mice

2. *In-vitro*: We examined factors from MRL that cause de-differentiation in culture. We used mouse myotubes and osteoblasts as mature cells.

3. *In-vivo*: We used the Swiss Webster (nonhealer) mouse, painted ear holes with compounds and looked for blastema formation. We also injected adenovirus constructs with FGF to achieve full growth of tail amputations.

Task 1.

To explore the ability of the MRL blastema to elicit transdifferentiation in mature populations of cells, cells were injected into the mouse ear, the ear punched a blastema formed and complete hole closure was seen. To identify the injected cells, we used male cells put into female mice. The male cells were detected by the use of Y chromosome in-situ hybridization. The first experiments using MRL male cartilage showed that after 1 month, transdifferentiation of male cells could be found in many tissues of the ear including mature muscle, cartilage, and dermal cells indicating a change within a cell type, all of these cells being mesenchymal. In addition, we saw an extensive number of donor origin Y-positive epithelial cells. This included basal epidermal cells as well as hair follicle cells. This represented a more extreme degree of transdifferentiation of cells from a mesenchymal to an epithelial cell type. Furthermore, a most striking finding was the development of a multicellular structure made up of different cell types being formed in the ear, almost resembling a patella.

The issue of which populations of cells are transdifferentiating, either mature cells or stem cells, could not be determined from these experiments. To address this issue and a second issue, whether nonsyngeneic cells could do the same in the ear, we used a clonal population of male endothelial cells from human. Since such cells would be rejected in a normal mouse, we made use of mice which were generated in the laboratory, immunodeficient MRL.RAG-/- mice, which we showed were equally regenerative. After 1 month, impressive transdifferentiation was seen in the MRL ears. Thus, the Y chromosome was detected in hair follicles and basal epidermal cells, indicating a cell type or mesenchymal to epithelial cell change.

Controls were C57BL/6 male cells injected into C57BL/6 female mice. We did not see transdifferentiation as was seen in the MRL mouse ear.

In conclusion, the MRL mouse appears to provide a permissive environment for transdifferentiation. One experiment that needs to be carried out is to determine at the single cell level if the Y-positive cells are not due to fusion but are in fact due to transdifferentiation.

Task 2:

To explore factors in-vitro derived from the MRL mouse responsible for blastema formation and transdifferentiation, we examined ear blastema extract which we added to mouse myotubes, multinucleated cells generated in culture from myoblasts. It had been shown by others that newt blastema extract when added to mouse myotube cultures led to 2 different events associated with de-differentiation. The first was re-entry into the cell cycle and this was measured by the uptake of BrdU. The second was the separation of the myotubes back into myoblasts.

We carried out similar experiments, however, we saw no effect of MRL blastema factor. We saw no BrdU uptake nor did we see separation of myotubes into myoblasts. Discussions with others have revealed that the presence of a stimulatory blastema factor appears quite early and we will examine this in the future.

We next tried MRL serum. We had been interested in MRL serum previously and it seemed worth using it in this system. We found that MRL serum when compared to C57BL/6 (the non-regenerating mouse strain) serum or fetal calf serum (FCS) showed BrdU incorporation, indicating entry intro S-phase of the cell cycle. The optimal concentration was 1% MRL serum. We found that the MRL serum had an effect on both MRL and C57BL/6 myotubes indicating that this was not only MRL serum-MRL cell specific. Furthermore, using live-cell microscopy, we showed that MRL myotubes do separate into myoblasts when exposed to MRL serum but not to C57BL/6 serum (data not shown).

Besides myotubes, we obtained the human osteosarcoma cell line SASOP57. This cell line stains for alkaline phosphatase. The degree of staining is related to the degree of differentiation so that upon de-differentiation, there is less alkaline phosphatase staining. We then grew these cells in different sera. These cells grown in MRL serum showed greater growth and the beginnings of the formation of a secondary structure, perhaps bone. Furthermore, the cells showed incredible cytoskeletal changes as seen by spicule formation. However, we did not see less staining. In fact, we saw more staining. Whether, the incubation period with serum is important is being examined.

Finally, we began to examine serum protein differences. We compared MRL and C57BL/6 serum by 2-D gel analysis and showed that there were many differences. However, since the MRL and C57BL/6 are very different mouse strains, we also examined the serum from a congenic mouse which we have developed. This mouse is a superhealer but >99% of its genome is derived from the C57BL/6 non-regenerating mouse and <1% is derived from MRL. When comparing the 3 serum samples, we found at least 4 spots that were present in the MRL and congenic but not the C57BL/6. Work will be done to identify these proteins as well as to separate the serum based on function.

We have accomplished the goals of this milestone. Thus, we have demonstrated an effect of an MRL factor present in MRL serum which can cause de-differentiation and proliferation. We believe that these are properties *in-vitro* that we have demonstrated in *in-vivo* and thus the re-capitulation of the events in the MRL.

Task 3.

In this task, we have attempted to recreate the regeneration seen in the MRL in a non-regenerating strain. We have been able to nearly close the ear hole of the non-regenerating strain, the Swiss Webster (SW) mouse. This was done by inducing an inflammatory response with a potent matrix metalloproteinase (MMP) component. This supports our prediction that the breakdown of the basement membrane would lead to fibroblast growth and regeneration. The fibroblast growth however was not very robust and we decided to use fibroblast growth factor (FGF) during the regenerative response. We used an adenovirus 5 expression vector with bFGF. We injected this into the tail and the ear hole of the SW mouse. We have seen both tail growth and ear hole closure. In the case of the tail, the blunt end of the severed tail becomes more normal looking and more pointed and grows in length. The results are positive but not sufficient to get complete re-growth. Also, the FGF vector may only be active for 1 week and multiple treatments may be necessary.

Publications: Data was presented at the Regenesis meeting in San Diego and at the seedling meeting in Va. Furthermore, the abstracts from the San Diego meeting will be published in "*Wound Repair and Regeneration*".

Future Plans: Since the funding for this project ended on December 30, 2003, further studies must wait for further funding. We are applying for further support from DARPA.