Research in Oral Sciences
From the submaxillary gland-saliva: Epidermal Growth Factor & Regenerative Medicine

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During the course of our studies on the nerve-growth promoting protein of the mouse sub-maxillary gland, it was noted that the daily injection of partially purified extracts … into new-born mice resulted in a number of gross anatomical changes in addition to the previously reported effects on the nerve cells.
This story dates from the “Bizzozzero paradigm” on cell renewal.

My speech on...

1. The Giulio Bizzozzero paradigm [growth factors and renewing/no renewing cells]
2. Receptors [growth factors and receptors]
3. No renewing cells: teeth, brain, heart: the [teeth, brain, heart] cells we have now are those we were born? [which source may be used for the replacement of dead cells: embryos, bone marrow, cord blood, amniotic fluid, adult stem cells?]
Giulio Bizzozzero: cell renewal: labile cells, stable, permanent/no renewing

- **Giulio Bizzozzero** [1846-1901, Turin Univ] introduced cell classification:
  - labile/renewing cells (e.g. bone marrow)
  - stable/normally no renewing/slow renewing cells (e.g. liver)
  - permanent/no renewing cells (e.g. nervous, heart)
1956-1965: breaking the dogma of permanent-no renewing cells: discovery of growth factors

- Rita Levi-Montalcini & Pietro U Angeletti, NGF [1958]
- Stanley Cohen: EGF [JBC 1962]
- Luigi Frati: G-CSF [BBA 1965]
- 1956 Nerve Growth Factor
- 1962 Epidermal Growth Factor
- 1965 Granulocyte GF [G-CSF]
NGF [soluble peptide] induces sensory chick embryo ganglia outgrowth [fiber halo]  
[Levi Montalcini R& Angeletti PU, Quart Rev Biol 1961; 36:99]  
A diffusible protein stimulates a specific fibers growth [sensory ganglia +++, epithelial cells -, bone marrow -]
50 years ago… Nov 20, 1961 Stanley Cohen sent a paper to the *Journal Biol Chem*

During the course of our studies on the nerve-growth promoting protein of the mouse sub-maxillary gland, it was noted that the daily injection of partially purified extracts … into new-born mice resulted in a number of gross anatomical changes in addition to the previously reported effects on the nerve cells.
During the course of our studies on the nerve-growth promoting protein of the mouse submaxillary gland, it was noted that the daily injection of partially purified extracts … into new-born mice resulted in a number of gross anatomical changes in addition to the previously reported effects on the nerve cells.

These were (a) precocious opening of the eyelids (as early as 7 days instead of the usual 12-14 days), (b) precocious eruption of the teeth (at the 6 to 7 days instead of the normal 8 to 10 days), and (c) a marked stunting of the animals with an inhibition of hair growth…
EGF is purified from a crude extract of submaxillary gland [S. Cohen, 1962]

**Fig. 1.** A, Chromatographic elution pattern of Fraction CM-1 on a CM-cellulose column with a sodium chloride gradient (see text); B, elution pattern of Fraction CM-2b during gel filtration on a column of Sephadex G-75 (see text); C, chromatographic elution pattern of the Sephadex fraction on a DEAE-cellulose column with a sodium chloride gradient (see text).

S Cohen. Isolation of a Mouse Submaxillary Gland Protein Accelerating Incisor Eruption and Eyelid Opening in the New-born Animal
EGF: protein of 6,200-12,000 [dimeric] daltons [S. Cohen, *JBC* 1962]

Stanley Cohen, JBC [1962]: the paper described EGF...

...a protein which elicits precocious eyelid opening and teeth eruption

S. Cohen, JBC 1968: EGF aa sequence – 53 aa, 3 -SS- bonds

EGF stimulates DNA, RNA and protein synthesis [HeLa cells] [L. Frati et al, 1972]

**Fig. 3.** Time-course of uptake of $^3$H-thymidine into HeLa cells incubated in the absence (●) and presence (○) of EGF (0.65 μg/ml).

**Fig. 4.** Uptake of $^3$H-uridine, $^{14}$C-amino acids and $^3$H-thymidine into KB cells, 10 h after start of incubation. □ control; ■ EGF (0.65 μg/ml).

EGF stimulates corneal epithelium healing
[1970]

EGF stimulates corneal epithelium healing [1970]: left EGF treated, right control [green fluorescence]

EGF stimulates corneal epithelium healing
[Frati L. et al, 1972]


- **green area**: scarification
- **left**: EGF treated, **healing** within the 4th day
- **right**: control, healing in progress [completed in 8-9 days]
Labeled EGF is bound to epidermis and corneal epithelium, this used to isolate the receptor

TABLE 3

Binding of EGF to mouse tissues (nmoles/mg wet tissue)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Binding (nmoles/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corneal epithelum</td>
<td>273 ± 112</td>
</tr>
<tr>
<td>Epidermis</td>
<td>145 ± 74</td>
</tr>
<tr>
<td>Submaxillary gland</td>
<td>0.08</td>
</tr>
<tr>
<td>Liver</td>
<td>0.23</td>
</tr>
<tr>
<td>Parotid</td>
<td>0.15</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.03</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.15</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.21</td>
</tr>
<tr>
<td>Brain</td>
<td>0.07</td>
</tr>
</tbody>
</table>

EGF-receptor is found on corneal epithelium [Frati L et al., Life Sciences 1976]

FIG. 1

Standard curve for the Epidermal Growth Factor radioreceptor assay. Experimental conditions are reported in the text.

Structure of the EGF-receptor [transmembrane protein], TK activity [S. Cohen, 1978]

Graham Carpenter, Stanley Cohen.
Epidermal growth factor.
Tyrosine Kinase activity and signal transduction [S. Cohen, 1978]

Graham Carpenter, Stanley Cohen.
Epidermal growth factor.
EGF stimulates DNA-RNA-protein synthesis on cancer HeLa cells [Henrietta Lack cancer cells]

**Fig. 3.** Time-course of uptake of $^3$H-thymidine into HeLa cells incubated in the absence (●) and presence (○) of EGF (0.65 µg/ml).

**Fig. 4.** Uptake of $^3$H-uridine, $^{14}$C-amino acids and $^3$H-thymidine into KB cells, 10 h after start of incubation. □ control; ■ EGF (0.65 µg/ml).

From the Nobel Foundation Directory: 1986 laureates on Physiology-Medicine

Stanley Cohen and Rita Levi-Montalcini

1986 COHEN, STANLEY, U.S.A., Vanderbilt University School of Medicine, Nashville, TN, * 1922; and

LEVI-MONTALCINI, RITA, Italy and U.S.A., Institute of Cell Biology of the C.N.R., Rome, Italy, * 1909 (in Turin, Italy): "för deras upptäckter av tillväxtfaktorer”; ”for their discoveries of growth factors".
The questions…

- EGF stimulates both normal and cancer cells
- EGF induces also differentiation
- EGF “family” [EGF-like molecules]
- EGF receptor “family”
- May we use EGF [other growth factors] to induce growth of dormant cells?
Growth Factors stimulate cell division; they are *stupid* molecules…

The problem is complicated by…

- Growth Factors [GF] and growth factors-like molecules: *cross reactivity-binding*

- Receptors are sensitive to families of GF [less or more]: *cross action*

- Growth Factors and their receptors: *relationship with oncogenes* [e.g. EGFr, *Notch*]
EGF-\textit{family} molecules
[disulphide bonds; peptide length: 40-60 aa]

EGF, both \textit{in vivo} and \textit{in vitro}, stimulates growth and differentiation through EGF\textsubscript{r} [\textit{“family”} of receptors]
EGF receptors family: dimerization-TK activation, effects on: cell survival, growth, etc.
### ErbB [EGF receptor] in human cancer: molecular diagnosis/prognosis vs therapy

<table>
<thead>
<tr>
<th>molecule</th>
<th>dysregulation</th>
<th>cancer; clinical use</th>
</tr>
</thead>
<tbody>
<tr>
<td>ErbB-1</td>
<td>overexpress/amplification</td>
<td>Breast: recurrence, survival; other [ovary, head-neck, bladder, prostate, kidney]: prognostic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glioma [40%]: grading, reduced survival</td>
</tr>
<tr>
<td></td>
<td>deletion-truncated</td>
<td>Breast, ovary, glioma, lung: reduced survival</td>
</tr>
<tr>
<td>ErbB-2</td>
<td>overexpress/amplification, homodimer active</td>
<td>Breast, ovary, cervix, endometrium, oesophagus, lung, pancreas, colon: grading, cell spreading, lack steroid receptors, antiestrogens resistance</td>
</tr>
<tr>
<td>ErbB-3</td>
<td>expression</td>
<td>Breast (co-expressed with ErbB-2 or 3), colon, gastric, prostate</td>
</tr>
<tr>
<td></td>
<td>overexpression</td>
<td>Oral squamous cancer: lymph nodes, survival</td>
</tr>
<tr>
<td>ErbB-4</td>
<td>expression</td>
<td>Medulloblastoma, ErbB-2 co-expressed, survival</td>
</tr>
<tr>
<td></td>
<td>reduced expression</td>
<td>Breast, prostate: differentiated phenotype related</td>
</tr>
</tbody>
</table>
Epidermal Growth Factor: Stanley Cohen, JBC 1962
- EGF receptor found-isolated from various tissues [L. Frati et al., Europ J Biochem, 1972; radioreceptor assay: Life Sciences 1976]
- Truncated EGFr-erbB family expressed in >50% of solid tumors; truncated EGFr related to malignancy
**Notch signaling:** ancient-conserved mechanism of cell-to-cell communication; role in development

[Gordon WR et al, J Cell Sci 2008]

**EGF-like repeats**

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**Diagram:**

- **Notch receptors**
  - *Drosophila Notch*
  - Human NOTCH1
  - Human NOTCH2
  - Human NOTCH3
  - Human NOTCH4
  - *C. elegans LIN-12*
  - *C. elegans GLP-1*

- **Key elements:**
  - EGF-like repeats
  - LNR HD
  - RAM ANK
  - PEST
  - Atypical EGF-like repeat
  - Ca²⁺-binding EGF-like repeat
Notch3 transgenic mice
[overexpression of EGFr repeats]

• T cell Leukemia induction
• Wild type mice: long term survivors
• Transgenic [TG] mice: all leukemia-lymphoma; death occurring in 2-3 weeks
Phenotype of Ick-Notch3-IC mice

T-cell leuk

Thymocytes

wt

tg

pTαa

28S

Splenic T cells

wt

tg

pTαa

β-actin

Thymocyte differentiation

CD4+wt

tg

CD8+wt

tg

TCRβ

wt

tg

IL2Rα

wt

tg

Lymph

spleen

WT

tg

T cell leukemia lymphoma

Luigi Frati

Diana Bellavia et al. EMBO J.

13:3337, 2000

Heart regeneration medicine

Age (weeks)
Growth Factors and receptors: some conclusions [’80-’90 years]

- GF may induce proliferation of so-called permanent/no renewing cells (e.g. nervous ganglia/cells, heart cells???)
- Useful to manipulate cells [commitment, differentiation]
- Active on different type of cells [e.g. normal and tumor cells; embryo, adult, etc]
- GF vs receptors; overexpression/tumors
The question of “dormant”/no renewing cells… The heart case

1. the *Giulio Bizzozzero* paradigm [growth factors and renewing/no renewing cells]
2. the receptors [growth factors and receptors]
3. The heart: *the heart cells we have now are those we were born?* [which source may be used for the replacement of heart dead cells? embryos, bone marrow, cord blood, amniotic fluid, adult stem cells?]
Taking the cells at various stages... embryo, cord blood, adult; toti- pluri- multi-potent; adult stem
Heart cell-therapy. Questions…

Which source? From…

- Embryos? No… Risk of tumors, modest therapeutic effects in animals, none in humans [01]
- Bone marrow
- Adult tissues
Heart cell-therapy. Questions…

Which source? From…

• Embryos? No…

• Amniotic fluid/cord blood? No effective…

• Bone marrow? Large studies

• Adult tissues
From bone marrow ➔ blood cells and... other...
But... Transdifferention? Conserve the fate?
Milestone 1 [Anversa P.]: Ventricle myocytes division in infarcted rats – but at low rate...

Myocytes nuclear mitotic divisions occur in the region adjacent to [and also distant from] the infarcted area of the left ventricle in rats 7 days after coronary occlusion.

Milestone 2 [Anversa P]: Y-chr cell from female recipient found in the [X-chr] male transplanted heart: cell migration into the heart from…? From bone marrow? Anversa studies…
Milestone 3 [Orlic & Anversa]: Bone marrow cells [BMCs] regenerate myocardium

Myocardial infarct (MI): $\text{Lin}^-/\text{c-}kit$ cells taken from bone marrow [arrows in top fig: regenerating myocardium; asterisks: necrotic myocytes; red: cardiac myosin; green: labelled nuclei]

Anversa P: no results by using “normal” BM cells; transgenic mice for β–actin into BM cells

[The P. Anversa group: M Rota/E Musso; K Urbanek & F Quaini; Jan Kajstura; AR Leri; EH Sonnenblick; SR Houser; R Bolli; M.A. Sussman]
Uncertain clinical results by using BM cells: why? Heart fate lost in translation!!!

- Uncertain results; why? Nature 2004; 428
- Murry CE, p. 664: BM derived SCs do not transdifferentiate; Balsam LB, p. 668: c-kit enriched BM cells injected into ischaemic myocardium express only CD-45 [haematological marker] – Anversa is adding a scar to a scar…
- Note: no heart specific but haematological/unspecific markers: kit+, Sca1+, SP, CD45+, CD133+;
- specific: troponin I, myosin, actin, CD 31, CD 105

a. Murry CE, p. 664: BM derived SCs do not transdifferentiate  

b. Balsam LB, p. 668: c-kit enriched BM cells [green fluorescent] injected into ischaemic myocardium express only CD-45 [haematological marker]
2004 turning point: heart adult stem cells isolated and cultured [cardiospheres]

Isolation and Expansion of Adult Cardiac Stem Cells From Human and Murine Heart
Elisa Messina, Luciana De Angelis, Giacomo Frati, Stefania Morrone, Stefano Chimenti, Fabio Fiordaliso, Monica Salio, Massimo Battaglia, Michael V.G. Latronico, Marcello Coletta, Elisabetta Vivarelli, Luigi Frati, Giulio Cossu and Alessandro Giacomello

Circ. Res. 2004;95:911-921; originally published online Oct 7, 2004;
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The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/cgi/content/full/95/9/911

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>Explants (1)</th>
<th>Cardiosphere-forming cells (4)</th>
<th>Cardiospheres (5)</th>
<th>Cardiosphere-derived cells (CDCs, 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image 1" /></td>
<td><img src="image2.png" alt="Image 2" /></td>
<td><img src="image3.png" alt="Image 3" /></td>
<td><img src="image4.png" alt="Image 4" /></td>
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<td><img src="image7.png" alt="Image 7" /></td>
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</tbody>
</table>

**Notes:**
- Explants (1) are isolated from biopsy samples.
- Cardiosphere-forming cells (4) are derived from explants.
- Cardiospheres (5) are harvested from cardiosphere-forming cells.
- Cardiosphere-derived cells (CDCs, 6) are derived from cardiospheres.

**Figure Legends:**
- Biopsy: Sample collected for analysis.
- Explants: Initial tissue samples.
- Cardiosphere-forming cells: Cells capable of forming cardiospheres.
- Cardiospheres: Rounded clusters of cells.
- Cardiosphere-derived cells (CDCs): Cells derived from cardiospheres.

**Images:**
- Image 1: Biopsy sample.
- Image 2: Explants (1).
- Image 3: Cardiosphere-forming cells.
- Image 4: Cardiospheres.
- Image 5: Cardiosphere-derived cells (CDCs).

**Cells:**
- c-Kit
- CD133
- CD105
- CD90

**Cell Counts:**
- 2,3 cardiosphere-forming cells
- 2,3 cardiosphere-derived cells (CDCs)
The method may be applied to humans: routine percutaneous heart biopsy.
Stable heart cells? Markers? C-kit, CD31, CD105, troponin I, myosin, actin...? **Cardiospheres beat...**
Stable heart cells? Markers? Spontaneous rhythmic electrical activity in a cardiosphere...

Pig CSps + NRVM day 6

-60 -40 -20 0 20 40

V_m (mV)

0 1 2 3 4 5 6

Time (s)
CDCs improve LV function post-MI in SCID mice: 4 weeks: contractions of whole LF

CDC-injected 4 weeks

Fibroblast-injected 4 weeks
Autologous human heart adult stem cells grow on bio-matrix to be used as patch on the infarcted heart

<table>
<thead>
<tr>
<th>Macro-scale</th>
<th>Micro-scale</th>
<th>Nano-scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Random</td>
<td>Nano fiber</td>
</tr>
<tr>
<td>B</td>
<td>Alignment</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Multi-layer</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Freeze-drying</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>SC-PL</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Gas foaming/SC-PL</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>Nano topology</td>
<td></td>
</tr>
</tbody>
</table>

Biomaterials

A. Electrospinning
- PLA, PGA, PLGA, PCL
- Collagen, gelatin, alginate, etc.
- Composite with CNT, DNA, and proteins

B. Liquid-solid transition
- Polymeric materials
- Hydrogel materials
- Composite materials

C. Releasing biomolecules
- Growth factors with polymeric carriers
- DNA with viral or non-viral carriers
Quantitative assessment with luciferase: patch bio-matrix reassorption

Day 1

Day 7

Day 21

Sacrifice

ROI 1 = 4.4755e+05

ROI 1 = 2.0649e+05

ROI 1 = 1.7377e+05

ROI 1 = 31040

Luigi Frati Regenerative Medicine
EGF and teeth eruption: 50 years ago started regenerative medicine. A dream-tooth…

S Cohen, Isolation of a Mouse Submaxillary Gland Protein Accelerating Incisor Eruption and Eyelid Opening in the New-born Animal
Thanks for your attention